AD_____

GRANT NUMBER DAMD17-97-1-7337

TITLE: Regulation Of The Tumor Suppressor Activity Of p53 In Human Breast Cancer

PRINCIPAL INVESTIGATOR: James J. Manfredi, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai Medical Center
New York, New York 10029-6574

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Aug 97 - 1 Aug 98)

4. TITLE AND SUBTITLE
Regulation Of The Tumor Suppressor Activity Of p53 In Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7337

6. AUTHOR(S)
James J. Manfredi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai Medical Center
New York, New York 10029-6574

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This research is centered on testing the hypothesis that there are novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration. The immediate goal of this research is to characterize cellular activities which affect the ability of p53 to bind to DNA in a sequence-specific manner. The long term goal is to determine the relevance of such regulators in human breast cancer. Human breast tumor samples would be screened for alterations in the expression of these proteins which affect the DNA binding activity of p53. The identification of proteins which regulate wild-type p53 is an important focus for breast cancer research since the regulation, mechanism of action, and metabolism of such proteins would be central to our understanding of breast cancer and the aberrant expression of such proteins would represent novel important mechanisms of carcinogenesis.

14. SUBJECT TERMS
Breast Cancer
p53, tumor suppressor, DNA binding

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

JJM For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JJM In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JJM In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JJM In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

James J Manfredi 8/28/98

PI - Signature Date

TABLE OF CONTENTS

Publications resulting from this research:

1. Resnick-Silverman, L., S. St Clair, M. Maurer, K. Zhao, and J. J. Manfredi. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. Genes Dev **12:**2102-7.

2. Tang, H. Y., K. Zhao, J. F. Pizzolato, M. Fonarev, J. C. Langer, and J. J. Manfredi. 1998. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. J Biol Chem **273:**29156-63.

ANNUAL REPORT - YEAR 1
Regulation of the tumor suppressor activity of p53 in human breast cancer
IDEA
(08/01/97-07/31/98)

INTRODUCTION

Genetic alteration of p53 resulting in loss-of-function is a common event in human cancer. This research is centered on testing the hypothesis that there are novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration. Consistent with its function as a transcription factor, the ability to bind to DNA has been shown to be central to the tumor suppressor activity of p53. Thus, the immediate goal of this study is to identify and characterize activities in human cells which affect the ability of p53 to bind to DNA in a sequence-specific manner. Two such activities have been found. The first stimulates the ability of p53 to bind to its sequence-specific binding site. The protein responsible for this activity is the high mobility group protein HMG-1. A second activity has been identified which binds in a sequence-specific manner to p53 binding sites, but is not p53 or one of the known p53 family members. Preliminary studies have suggested that this factor can compete with p53 for binding to DNA. It is proposed that such an activity could interfere with the interaction of p53 with its target genes and thereby inhibit the ability of p53 to activate gene expression. As the protein(s) that are responsible for this inhibitory activity are still unknown, biochemical purification and identification of the constituent polypeptides for this activity are underway. The activity will be purified by a variety of biochemical means with the goal of generating either amino acid sequence data or antibodies to allow for cloning of the relevant effector polypeptides. Subsequently, the genes that encode the constituent polypeptide(s) will be cloned and the resulting gene products will be characterized. Two approaches have been proposed. First, microsequencing of purified proteins will be used to generate oligonucleotide probes for screening. Second, antibodies will be made to these purified proteins and used to screen a prokaryotic expression library.

The long term goal is to determine the relevance of both the enhancing activity, identified as HMG-1, and this inhibitory activity, as yet uncharacterized, in human breast cancer. Human breast tumor samples would be screened for alterations in the expression of these factors which affect the DNA binding activity of p53. The identification of proteins which regulate wild-type p53 is an important focus for breast cancer research since the regulation, mechanism of action, and metabolism of such proteins would be central to our understanding of breast cancer and the aberrant expression of such proteins would represent novel important mechanisms of carcinogenesis.

BODY

Results

Further characterization of nuclear extract from Saos-2 cells has identified two activities which influence the ability of p53 to bind to DNA in a sequence-specific

manner. The first is the high mobility group protein, HMG-1, and the second is an, as yet uncharacterized, factor which can inhibit the binding of p53 to a subset of its binding sites. Preliminary studies involving nuclear extracts derived from several different cell lines identified a cellular factor which bound to synthetic p53 consensus binding site but did not appear to be p53. This data was published in the Journal of Biological Chemistry as part of a larger study (18). The studies described here further characterize two novel cellular factors which bind in a sequence-specific manner to only a subset of p53 binding sites.

High mobility group proteins. In an attempt to further characterize p53EF, several known factors which have been shown to enhance the DNA binding of other transcription factors were examined. Among these, it was found that three of the high mobility group proteins, HMG-1, HMG-2, and HMG-I(Y), all were capable of stimulating the sequence-specific DNA binding activity of p53 *in vitro*, but only HMG-1 was capable of enhancing the transcriptional activity of p53 in cells. The high mobility group proteins were each expressed in bacteria and purified. Human p53 protein was purified from recombinant baculovirus-infected insect cells. Electrophoretic mobility shift assays were performed using as radiolabeled probes each of the two p53 response elements from the human *p21* promoter. Incubation of increasing amounts of either HMG-1, HMG-2, or HMG-I(Y) enhanced the ability of p53 to bind to either of these sites. Plasmids expressing each of these HMG proteins were transfected into p53-null Saos-2 cells with an expression vector for p53 and luciferase reporters constructs that contain either of the p53 sites from the *p21* promoter upstream of a minimal adenovirus E1b promoter. Expression of HMG-1 but not HMG-2 or HMG-I(Y) stimulated the ability of p53 to transcriptionally activate via either of these sites. This latter result suggests that additional levels of regulation occur in the intact cell such that only HMG-1 affects p53-dependent transcription.

As already noted, two p53 binding sites in the human *p21* promoter have been identified which mediate the transcriptional responsiveness of this promoter to p53. The sequence of one of these, the 5′ site, matches well to the previously identified consensus for p53 binding with C residues in the fourth position of each of the four pentamers which make up the p53 binding site. The binding of p53 by a monoclonal antibody, mAb 421, enhances the ability of p53 to bind to this site. The sequence of the second site, the 3′ site, contains a G in place of a C residue in the fourth position of one pentamer. Interestingly, the binding of mAb 421 to p53 inhibits the ability of p53 to bind to this site. Mutational analysis showed that a single base change can cause one site to behave similarly to the other site. A response element in the promoter of the human *cdc25C* gene has been identified to which p53 bound with similar properties as the 3′ site. These results demonstrate the existence of two classes of p53 binding sites in the human genome and that the binding of p53 to these two classes of sites can be differentially regulated by binding of mAb 421. This is a novel example of the regulation of binding site selection by a transcription factor and suggests a possible mechanism for selectivity in target gene activation by the p53 protein. These studies resulted in a publication in Genes and Development (15) (see Appendices).

Electrophoretic mobility shift assays in which HMG-1 was added to DNA binding mixtures containing mAb 421 demonstrated that HMG-1 can also enhance the ability of p53 to bind to both the *p21* 5′ site and the *p21* 3′ site in the presence of mAb 421 However the effect of HMG-1 was much more dramatic on the binding of p53 to the *p21* 3′ site than the *p21* 5′ site. Thus, quantitation of the EMSA results showed that in the

absence of mAb 421, HMG-1 enhanced the binding of p53 to either the p21 5′ site (ten-fold) or to the p21 3′ site (three-fold). In contrast, in the presence of mAb 421, the enhancement by HMG-1 on binding to the 5′ site was slight (1.3 to 1.4-fold) whereas the enhancement of binding to the 3′ site was six-fold. These results suggest that the presence of HMG-1 not only enhances the binding of p53 to both sites in the presence of mAb 421, but that, in fact, HMG-1 can reverse the inhibition of binding of p53 to the 3′ site that is seen in the presence of mAb 421.

These EMSA results may merely reflect the ability of HMG-1 to enhance the DNA binding of p53 under all conditions, rather than specifically reverse the inhibitory effect of mAb 421. To directly address this question, a level of HMG-1 protein was used in EMSA studies which did not measurably enhance the DNA binding of p53 to either the 5′ site or the 3′ site. Thus, a range of HMG-1 from 20-100 ng was insufficient to enhance the binding of p53 to either the 5′ or the 3′ site. Nevertheless, this level of HMG-1 protein was capable of relieving the inhibitory effect of mAb 421 on the binding of p53 to the 3′ site. Incubation with 60 ng of HMG-1 enhanced the supershifted complex whereas it had no apparent effect on the binding of p53 to the 3′ site in the absence of mAb 421. In contrast, there was a minimal effect of this level of HMG-1 on the binding of p53 to the 5′ site in the presence of mAb 421. Addition of this level of HMG-1 to the p53 reaction mixes containing mAb 421 with the 5′ site as probe did not increase the amount of supershifted complex. These results suggest that the ability to relieve the mAb 421 inhibitory effect and enhancement of DNA binding are separable functions of HMG-1. This is a direct demonstration that the ability of HMG-1 to enhance the binding of p53 to DNA under a variety of condition can be distinguished from the specific ability of HMG-1 to reverse the ability of mAb 421 to inhibit the binding of p53 to the 3′ site. Since reversal of the mAb 421 inhibition occurs at much lower levels of HMG-1 than enhancement of DNA binding, the results presented here are consistent with the possibility that catalytic amounts of HMG-1 may be required for one function (reversal of inhibition by mAb 421) whereas stoichiometric amounts may be necessary for the other (enhancement of DNA binding).

BOB1 and BOB2. Electrophoretic mobility shift assays were performed with nuclear extract from p53-null Saos-2 cells using the p53 element from the human bax promoter as radiolabeled probe. Four distinct complexes were identified. Competition experiments demonstrated that two of these complexes were non-specific. The two specific complexes were referred to as bob1 and bob2 (binder of bax). These complexes were unaffected by the presence of anti-p53 antibodies. Further, bob1 and bob2 failed to bind to the upstream element from the *p21* promoter nor did they bind to oligonucleotides corresponding to the p53 response elements of the *gadd45* gene and the downstream element of the *mdm-2* gene. As all of the currently identified p53 family members appear to interact with DNA with a similar sequence specificity to that of p53 itself, it can be argued that bob1 and bob2 represent novel factors. Increasing amounts of nuclear extract will inhibit the binding of purified p53 to DNA and similarly increasing amounts of purified p53 will block the formation of the bob1 and bob2 complexes. This suggests the intriguing possibility that bob1 and bob2 may bind to a subset of p53 binding sites and block the interaction of p53 with those elements.

Discussion

HMG-1. HMG-1 and 2 belong to a highly conserved family of abundant chromatin-associated nucleoproteins that have shown to both bind and bend DNA (1, 4, 11-13). These features have been associated with their apparent ability to enhance the activity of transcription factors. Sequence-specific activation of the progesterone receptor (11), the estrogen receptor (19), HOX proteins (21), and Oct-1, -2 and -6 proteins (23) have been reported in the literature. Although HMG-1 and -2 do not exhibit sequence-specific binding to DNA, it is postulated that they recognize architectural features of DNA such as palindromes, B-Z DNA junctions, cruciforms, and stem-loops. HMG-I(Y), on the other hand, prefers to bind to A/T-rich tracts of DNA (1) but there is also *in vitro* evidence of HMG-I(Y) binding to cruciform structures (2, 22), non-B-form structures in supercoiled plasmids (10) and distorted regions of DNA found on nucleosome core particles (14). Thus, it was surprising that HMG-I(Y) behaved similarly to HMG-1 and -2 in their effects on the *in vitro* DNA binding of p53. Since these sites do not contain A/T tracts, the mode of interaction might indeed be architectural rather than sequence specific. The palindromic nature of the consensus sequence may provide structural recognition for the HMG proteins.

The amount of HMG protein required for reversal of the mAb421 effect on the 3′ site was insufficient for enhancement of binding of p53 to these sites. These results suggest that there may be two distinct mechanisms that are in operation. Recent evidence that HMG-1 binds directly to p53 may help to explain these results (6). One can postulate that stoichiometric amounts of HMG-1 may be required to interact with p53 yet lesser catalytic amounts may be sufficient to relieve the mAb421 inhibition effect.

Although HMG-1, -2 and -I(Y) had similar effects on the DNA binding reaction *in vitro*, transfections in cells yielded different results for these three HMG proteins. Only HMG-1 enhanced the p53 dependent transactivation of the reporter vectors. This specificity may be apparent only in a transfection assay because of the presence of undefined cellular factors that may be required for transactivation.

BOB1 and BOB2. Preliminary results indicated that the binding of p53 and bob1 or bob2 to this p53 response element were mutually exclusive, suggesting that these factors may actually compete with p53 for binding. Elucidation of the roles of these factors in p53-mediated transactivation must await the cloning of their respective genes and the direct examination of their interactions with p53 response elements, as well as the mechanisms regulating their functions. One can speculate, nonetheless, that these factors may modulate p53 function by competing with p53 for binding to a subset of p53 response genes, in particular, *bax*. If this is indeed the case, it would represent a novel mechanism for regulating the function of the tumor suppressor p53. To date, it has been shown that the ability of p53 to bind DNA can be regulated by phosphorylation (17, 20), acetylation (16), and sequestration both by cellular and viral proteins (3, 5, 7-9). This would be the first example showing that the binding of p53 to DNA also is regulated by a competitive inhibitor. Further, if these factors do compete with p53 for binding to the *bax* promoter, one could hypothesize that alterations in the activities of these factors may explain the reported differential regulation of p53-mediated transactivation of *bax* as compared to other target genes.

Adherence to Statement of Work

Year 1
Experiments to be performed in Year 1 were completed as planned. Cellular factors have been identified and partially characterized.

Year 2
Purification of the two factors, bob1 and bob2, are underway as planned for Year 2.

CONCLUSIONS

The goal of the research contained within this proposal was to characterize cellular factors which regulate the interaction of p53 with its sequence-specific binding sites. Two such factors have been studied. The first is an activity which enhances the DNA binding activity of p53 and has been identified as being the high mobility group protein HMG-1. The second is an, as yet unidentified, activity which inhibits the binding of p53 to a subset of p53 response elements. The research supported by this funding has produced two publications (15, 18). In the coming year, the interaction of HMG-1 and p53 will be more fully characterized and the inhibitory factors will be purified and their constituent polypeptide(s) will be cloned. Characterization of both these sets of factors are an important first step in determining whether they play a role in affecting the tumor suppressor activity of p53 in human breast cancer.

REFERENCES

1. Bustin, M., and R. Reeves. 1996. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol **54**:35-100.
2. Claus, P., E. Schulze, and J. R. Wisniewski. 1994. Insect proteins homologous to mammalian high mobility group proteins I/Y (HMG I/Y). Characterization and binding to linear and four-way junction DNA. J Biol Chem **269**:33042-8.
3. Debbas, M., and E. White. 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev **7**:546-54.
4. Grosschedl, R., K. Giese, and J. Pagel. 1994. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet **10**:94-100.
5. Haupt, Y., S. Rowan, E. Shaulian, K. H. Vousden, and M. Oren. 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev **9**:2170-2183.
6. Jayaraman, L., N. C. Moorthy, K. G. Murthy, J. L. Manley, M. Bustin, and C. Prives. 1998. High mobility group protein-1 (HMG-1) is a unique activator of p53. Genes Dev **12**:462-72.
7. Kubbutat, M. H., S. N. Jones, and K. H. Vousden. 1997. Regulation of p53 stability by Mdm2. Nature **387**:299-303.
8. Lechner, M. S., D. H. Mack, A. B. Finicle, T. Crook, K. H. Vousden, and L. A. Laimins. 1992. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53- mediated repression of transcription. Embo J **11**:3045-52.
9. Lowe, S. W., and H. E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev **7**:535-45.

10. Nissen, M. S., and R. Reeves. 1995. Changes in superhelicity are introduced into closed circular DNA by binding of high mobility group protein I/Y. J Biol Chem **270:**4355-60.
11. Onate, S. A., P. Prendergast, J. P. Wagner, M. Nissen, R. Reeves, D. E. Pettijohn, and D. P. Edwards. 1994. The DNA-bending protein HMG-1 enhances progesterone receptor binding to its target DNA sequences. Mol Cell Biol **14:**3376-91.
12. Paull, T. T., M. J. Haykinson, and R. C. Johnson. 1993. The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. Genes Dev **7:**1521-34.
13. Pil, P. M., C. S. Chow, and S. J. Lippard. 1993. High-mobility-group 1 protein mediates DNA bending as determined by ring closures. Proc Natl Acad Sci U S A **90:**9465-9.
14. Reeves, R., and M. S. Nissen. 1993. Interaction of high mobility group-I (Y) nonhistone proteins with nucleosome core particles. J Biol Chem **268:**21137-46.
15. Resnick-Silverman, L., S. St Clair, M. Maurer, K. Zhao, and J. J. Manfredi. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. Genes Dev **12:**2102-7.
16. Sakaguchi, K., J. E. Herrera, S. Saito, T. Miki, M. Bustin, A. Vassilev, C. W. Anderson, and E. Appella. 1998. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev **12:**2831-41.
17. Shieh, S. Y., M. Ikeda, Y. Taya, and C. Prives. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell **91:**325-34.
18. Tang, H. Y., K. Zhao, J. F. Pizzolato, M. Fonarev, J. C. Langer, and J. J. Manfredi. 1998. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. J Biol Chem **273:**29156-63.
19. Verrier, C. S., N. Roodi, C. J. Yee, L. R. Bailey, R. A. Jensen, M. Bustin, and F. F. Parl. 1997. High-mobility group (HMG) protein HMG-1 and TATA-binding protein- associated factor TAF(II)30 affect estrogen receptor-mediated transcriptional activation. Mol Endocrinol **11:**1009-19.
20. Woo, R. A., K. G. McLure, S. P. Lees-Miller, D. E. Rancourt, and P. W. Lee. 1998. DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. Nature **394:**700-4.
21. Zappavigna, V., L. Falciola, M. H. Citterich, F. Mavilio, and M. E. Bianchi. 1996. HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. Embo J **15:**4981-91.
22. Zeleznik-Le, N. J., A. M. Harden, and J. D. Rowley. 1994. 11q23 translocations split the "AT-hook" cruciform DNA-binding region and the transcriptional repression domain from the activation domain of the mixed-lineage leukemia (MLL) gene. Proc Natl Acad Sci U S A **91:**10610-4.
23. Zwilling, S., H. Konig, and T. Wirth. 1995. High mobility group protein 2 functionally interacts with the POU domains of octamer transcription factors. Embo J **14:**1198-208.

RESEARCH COMMUNICATION

Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53

Lois Resnick-Silverman,[1] Selvon St. Clair,[1] Matthew Maurer,[1] Kathy Zhao,[1] and James J. Manfredi[1-3]

[1]Derald H. Ruttenberg Cancer Center and the [2]Brookdale Center for Molecular and Developmental Biology, Mount Sinai School of Medicine, New York, New York 10029 USA

There are two response elements for p53 in the promoter of the gene for the cyclin-dependent kinase inhibitor p21. The binding of p53 to the 5′ site was enhanced by incubation with monoclonal antibody 421, whereas the binding of p53 to the 3′ site was inhibited. Mutational analysis showed that a single-base change caused one element to behave like the other. A response element in the human *cdc25C* promoter is bound by p53 with properties similar to the 3′ site. These results identify two classes of p53-binding sites and suggest a mechanism for target gene selectivity by p53.

Received March 25, 1998; revised version accepted May 27, 1998.

The tumor suppressor protein p53 has been implicated in the cellular response to DNA damage and mediates either growth arrest or apoptosis, depending on particular cellular conditions (see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997). It has also been implicated in a spindle checkpoint (Cross et al. 1995) and in the induction of either differentiation (Shaulsky et al. 1991; Aloni-Grinstein et al. 1993; Soddu et al. 1994) or senescence (Sugrue et al. 1997). The p53 protein is a transcription factor that binds in a sequence-specific manner to particular sites in the genome and activates transcription of target genes (for review, see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997). Utilizing immunobinding assays involving the monoclonal antibody 421, a consensus binding site for p53 has been defined and consists of four pentameric repeats of RRRCW in which R is a purine and W represents either an A or T residue (El-Deiry et al. 1992; Funk et al. 1992; Halazonetis et al. 1993). Two palindromic pentamers (half-site) are juxtaposed to a second set of two palindromic pentamers, the two half-sites being separated by no insert or insertions from 1–13 bp (El-Deiry et al. 1992). Such a consensus site is consistent with the fact that p53 exists in solution and binds to DNA as a tetramer (Friedman et al. 1993). It has been proposed that to accommodate a symmetrical tetrameric p53 on such a site, the DNA must bend (Balagurumoorthy et al. 1995; Nagaich et al. 1997a,b). Studies to date have implicated the C residue at position 4 of each pentamer as essential for the binding of p53 to DNA (Halazonetis et al. 1993; Nagaich et al. 1997b).

The structure of p53 is consistent with its role as a transcription factor with identified domains that are responsible for transcriptional activation, sequence-specific DNA binding, and oligomerization as a tetramer. Previous studies have implicated the carboxy-terminal 30 amino acids of p53 as exerting a negative regulatory effect on the DNA-binding activity of the protein. Deletion of these carboxy-terminal 30 amino acids, phosphorylation of sites within this region by casein kinase II and protein kinase C, and the binding of bacterial DnaK in this region all will activate the DNA-binding activity of p53 (Hupp et al. 1992; Takenaka et al. 1995). Consistent with this, the mAb 421, which has an epitope in this carboxy-terminal region, activates the ability of p53 to bind to DNA (Funk et al. 1992; Hupp et al. 1992; Halazonetis et al. 1993; Mundt et al. 1997). Finally, a peptide derived from the carboxyl end of p53 has also been shown to stimulate the ability of p53 to interact with DNA, although not to the same extent as the activators identified previously (Hupp et al. 1995).

DNA damage induces expression of p53 protein which, in turn, transcriptionally activates expression of particular genes, most notably those that encode the cyclin-dependent kinase inhibitor p21. Consistent with this, cells that lack p21 expression have an impaired p53-dependent response to DNA damage (Brugarolas et al. 1995; Deng et al. 1995). The human *p21* promoter has been shown to contain two p53-responsive elements. Deletion analysis of reporter constructs containing the sequence of the human *p21* promoter identified a distal element located 2.3–2.5 kb and a proximal element located 1.1–1.5 kb from the start site of transcription (El-Deiry et al. 1993,1995; Macleod et al. 1995).

In this report we have confirmed the existence of two p53-responsive elements in the human *p21* promoter. One of these, the 3′ site, matches the consensus sequence for p53 DNA binding at 18 of 20 positions. Notably, there is a G residue in place of the C residue in the fourth position of the first pentamer (Fig. 1). In contrast to other known p53-binding sites, the binding of p53 to this 3′ site in the *p21* promoter is inhibited by mAb 421. This suggests the existence of a new class of genomic sites in which the binding of p53 may be regulated differentially. Because p53 has been implicated in a variety of cellular responses, an understanding of the mechanism for selection of target genes by p53 is central to understanding its biological functions. The results pre-

[*Key Words:* Tumor suppressor; DNA binding; sequence specificity; p53 protein; transcriptional activation; binding sites]
[3]Corresponding author.
E-MAIL jmanfredi@smtplink.mssm.edu; FAX (212) 849-2446.

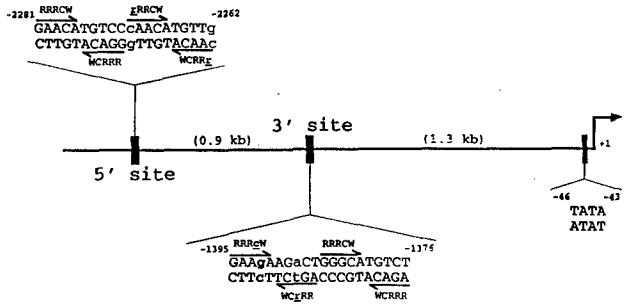

Figure 1. Schematic of *p21* promoter. Shown is 2.5 kb of the upstream sequence of the human *p21* promoter; 2.2 kb from the start site of transcription is a well-documented p53-binding site at positions -2281 to -2262. It matches the published consensus sequence for a p53 DNA binding site in 18 of 20 positions; the variations from the consensus are shown by lowercase letters. A second site with similarity to the consensus sequence is 1.3 kb upstream from the start site of transcription. Contained within positions -1395 to -1376, this sequence also matches the consensus at 18 of 20 positions. Note that in this 3′ site, the fourth position of the first pentamer contains a G rather than a C residue (shown in lowercase and boldface type). The position of the TATA box of the promoter is also indicated.

sented here suggest one potential mechanism for such site selection by p53.

Results

Two p53-responsive elements are present in the human p21 *promoter*

Using a series of deletion constructs, two p53-response elements had been identified previously in the human *p21* promoter (Macleod et al. 1995). A well-characterized element was located 2.4 kb upstream from the start site of transcription, and a second element had been suggested to be present 1.1–1.5 kb from the start of transcription (Fig. 1). We demonstrate that both a 20-bp 5′ element, located between -2262 and -2281, and a 20-bp 3′ element, located between -1376 and -1395, are each sufficient to transactivate a reporter gene in a p53-dependent manner (Fig. 2). Double-stranded synthetic oligonucleotides containing either one copy of the 5′ site or two copies of the 3′ site were inserted into a reporter vector, pGL3–E1bTATA, containing the *E1b* promoter upstream of a luciferase reporter gene. Although a single copy of the 3′ site conferred p53-dependent transcriptional activation on the minimal promoter (see Fig. 5B, below), two copies of the 3′ site showed a more pronounced effect and were used in the experiments described here (Fig. 2). Each reporter construct was cotransfected into p53-negative Saos-2 cells with empty vector or a plasmid expressing either human wild-type p53 or the human temperature-sensitive mutant $p53^{Ala143}$. The temperature-sensitive mutant $p53^{Ala143}$ is in a mutant conformation at 37°C. At this temperature, it is unable to activate p53-response elements. However, when shifted to 32°C, this mutant can assume a wild-type conformation and has been shown to activate some p53-responsive promoters (such as *p21*) but not others (such as Bax) (Friedlander et al. 1996). Cells were maintained at 37°C or shifted to 32°C, 17 hr prior to lysis. At 37°C, a luciferase reporter containing a single copy of the 5′ site was activated 1730-fold by wild-type p53, but only 2-fold by $p53^{Ala143}$. The reporter plasmid containing two copies of the 3′ site was activated 380-fold by wild-type p53, but again only 2-fold by $p53^{Ala143}$. The reporter vector lacking either response element was minimally activated by expression of either the wild-type or mutant p53 (Fig. 2A). At 32°C, the luciferase reporter containing a single copy of the 5′ site was activated 1154-fold by wild-type p53 and 792-fold in the presence of $p53^{Ala143}$. The reporter plasmid containing two copies of the 3′ site was activated 362-fold by wild-type p53 and 96-fold by the mutant $p53^{Ala143}$. A luciferase reporter plasmid containing the full-length *p21* promoter p21P was activated 16-fold by wild-type p53 and ninefold by the mutant $p53^{Ala143}$. At 32°C, the reporter vector lacking either response element was not activated by either wild-type or mutant p53 (Fig. 2B). These data confirm that there are two p53-response elements in the human *p21* promoter, each of which is sufficent to confer p53-dependent transcriptional activation on a luciferase reporter gene containing the minimal adenovirus *E1b* promoter. Additionally, neither the 5′ nor the 3′ site is activated by the temperature-sensitive mutant $p53^{Ala143}$ at 37°C, whereas at 32°C both sites are activated, although less so than by wild-type p53.

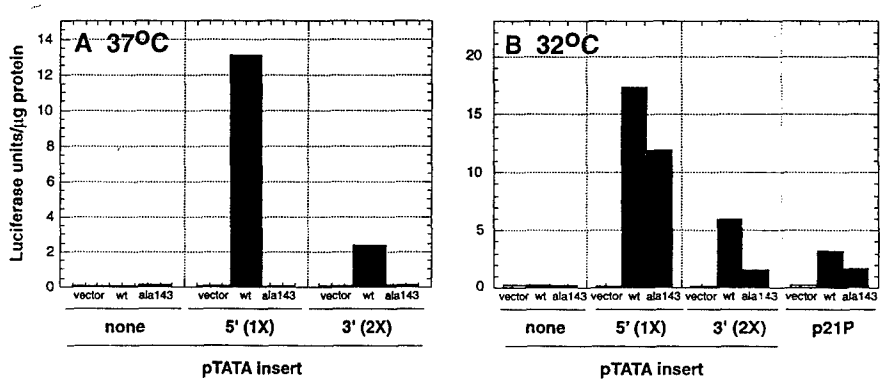

Figure 2. Two p53-response elements are present in the *p21* promoter. Saos-2 cells were transfected as described in Materials and Methods with 2 μg of the indicated reporter constructs in the absence or presence of either 50 ng of pCMV–p53wt, an expression vector encoding human wild-type p53 under the control of the CMV promoter (light and dark bars, respectively), or 50 ng of pCMV–$p53^{ala143}$, an expression vector encoding the temperature-sensitive mutant $p53^{Ala143}$ (solid bars). Cells were either maintained at 37°C (*A*) or shifted to 32°C 17 hr prior to lysis (*B*) and then were assayed for luciferase activity and total protein levels as described in Materials and Methods. The indicated values are the average of three (37°C) or four (32°C) independent experiments that had been performed in duplicate.

Two p53-binding sites are present in the human p21 *promoter*

Double-stranded oligonucleotides that contain the sequence of the 5′ and 3′ sites were synthesized and used in electrophoretic mobility shift assays (EMSAs). The 5′ site was bound by purified p53, and this binding was competed by an excess of unlabeled 5′ site (Fig. 3A, lanes 2–4), as well as an excess of the 3′ site (Fig. 3A, lanes 8–10), but not by an unlabeled oligonucleotide containing a mutated sequence of the 5′ site in which the C residue in the fourth position of each pentamer has been mutated to a T residue (Fig. 3A, lanes 5–7). The 3′ site competes approximately threefold less well than the 5′ site for the binding of p53 protein to a labeled 5′ site (Fig. 3A, lanes 2–4, and 8–10). The mAb 1801 supershifts the p53–5′ site complex efficiently, demonstrating the presence of p53 in that complex (Fig. 3A, lane 11). As has been reported previously for a p53 consensus site (Funk et al. 1992), mAb 421 also efficiently supershifts the p53–5′ site complex and in so doing enhances the binding that is seen (Fig. 3A, lane 12).

The 3′ site also was bound by purified p53, and the binding of p53 to a labeled 3′ site was effectively competed by the unlabeled 3′ site (Fig. 3B, lanes 2–4), as well as the unlabeled 5′ site (Fig. 3B, lanes 8–10) but not the mutated 5′ site with all four C residues altered (Fig. 3B, lanes 5–7). Consistent with the results of the competition analysis using the labeled 5′ site, the unlabeled 5′ site competed threefold better for binding to the labeled 3′ site as the unlabeled 3′ site (Fig. 3B, lanes 2–4, and 8–10). mAb 1801 again supershifted the p53–3′ site complex (Fig. 3B, lane 11) efficiently. Surprisingly, in contrast to the result with the labeled 5′ site, mAb 421 appeared to inhibit the binding of p53 to the 3′ site and supershifted what little DNA-binding complex that was detected only poorly (Fig. 3B, lane 12). This latter result suggests the intriguing possibility that the optimal binding of p53 to each of these sites may require different conformations of the p53 tetramer.

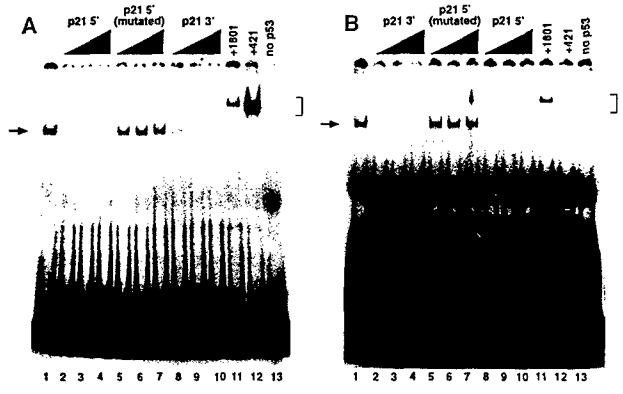

Figure 3. Monoclonal antibody enhances the binding of p53 to the 5′ site but inhibits the binding of p53 to the 3′ site. (*A*) An EMSA using as labeled probe the 5′ p53-binding site; (*B*) an EMSA using as labeled probe the 3′ p53-binding site. Five nanograms of purified p53 protein was incubated alone (lane *1*), in the presence of a 17-, 33-, or 50-fold excess of each unlabeled competitor, as indicated (lanes *2–10*), or in the presence of a 4 µl of mAb 1801 (lane *11*) or 4 µl of mAb 421 (lane *12*). p21 5′ (mutated) refers to a 5′ site in which the fourth postion of each pentamer has been mutated to a T residue. A sample that does not contain any p53 protein is shown in lane *13*. (→) The position of the p53–DNA complex; (]) the position of the supershifted p53–DNA antibody complex.

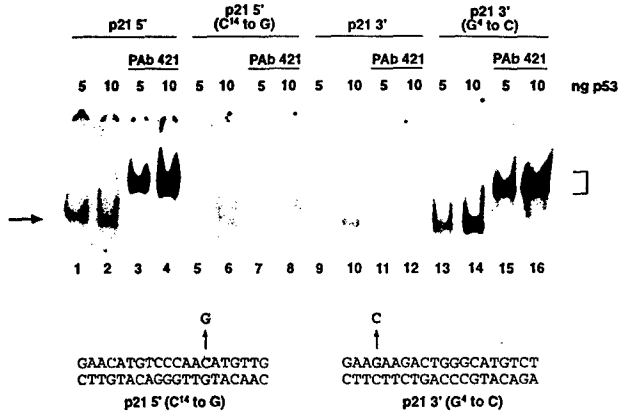

Figure 4. Mutational analysis demonstrates the importance of the C residue in the fourth position of a pentamer in responsiveness to mAb 421. Purified p53 (5 or 10 ng as indicated) was incubated with labeled probes containing the 5′ site (lanes *1–4*), the 5′ site with the residue at position 14 mutated to a G (C^{14} to G) (lanes *5–8*), the 3′ site (lanes *9–12*), or the 3′ site with the residue at position 4 mutated to a C (G^4 to C) (lanes *13–16*). The labeled probes had equivalent specific activities. Reactions were performed either in the absence (lanes *1,2,5,6,9,10,13,14*) or presence (lanes *3,4,7,8,11,12,15,16*) of mAb 421. The arrow (→) position of the p53–DNA complex; (]) position of the supershifted p53–DNA–antibody complex.

Mutation of a C residue affects responsiveness to mAb 421

The 3′ site diverges from the published consensus for p53-binding sites in two positions. The residue in position 4 of the first pentamer is a G (instead of a C residue), and the residue in position 3 of the second pentamer is an A residue (instead of a pyrimidine). The binding of p53 to the labeled 3′ site is inhibited in the presence of mAb 421 (Fig. 4, lanes 9–12). However, when a 1-bp change, G^4 to C residue, was engineered in this sequence and used as the labeled probe, the binding to p53 was enhanced in the presence of mAb 421 (Fig. 4, lanes 13–16). Thus, mutation of G^4 to a C, thereby creating a site with four consensus pentamers, allowed the binding of p53 to this site to be enhanced by mAb 421.

The reverse effect could be achieved through mutagenesis of the 5′ site. This site conforms to the consensus sequence in that a C residue is present in the fourth position of each pentamer. A labeled 5′ probe bound to increasing amounts of p53 (Fig. 4, lanes 1–2) and this binding could be enhanced in the presence of mAb 421 (Fig. 4, lanes 3,4). When mutated 5′ (C^{14} to G) was labeled and used in this EMSA, its binding to p53 was inhibited in the presence of 421 (Fig. 4, lanes 5–8) just like the 3′ site (Fig. 4, lanes 9–12). Therefore, the primary sequence of the response element can determine

whether the binding by p53 is enhanced or inhibited by antibody 421.

There is a p53-response element in the promoter of the human cdc25C *gene with properties similar to the 3′ site*

A search in the human genome database for other variant p53-binding sites that consist of four pentamers, only three of which contain C residues in the fourth position, was performed. A site in the promoter of the *cdc25C* gene, which encodes a cell cycle-regulated protein phosphatase that is necessary for progression into mitosis, was subjected to further analysis. A radiolabeled synthetic oligonucleotide containing a sequence from the human *cdc25C* promoter is bound by purified human p53 in an EMSA (Fig. 5A, lane 7). mAb 1801 supershifts this complex efficiently, whereas mAb 421 inhibits the binding of p53 to this site (Fig. 5A, lanes 8,9). Similar results were obtained with a radiolabeled oligonucleotide containing the sequence of the 3′ site of the *p21* promoter (Fig. 5A, lanes 4–6). These results are in contrast to the ability of mAb 421 to enhance and supershift the complex of p53 with a radiolabled oligonucleotide containing the 5′ site from the *p21* promoter (Fig. 5A, lanes 1–3). These results demonstrate that the site from the *cdc25C* promoter binds to p53 in the presence of mAb 421 with similar properties as the 3′ site from the *p21* promoter.

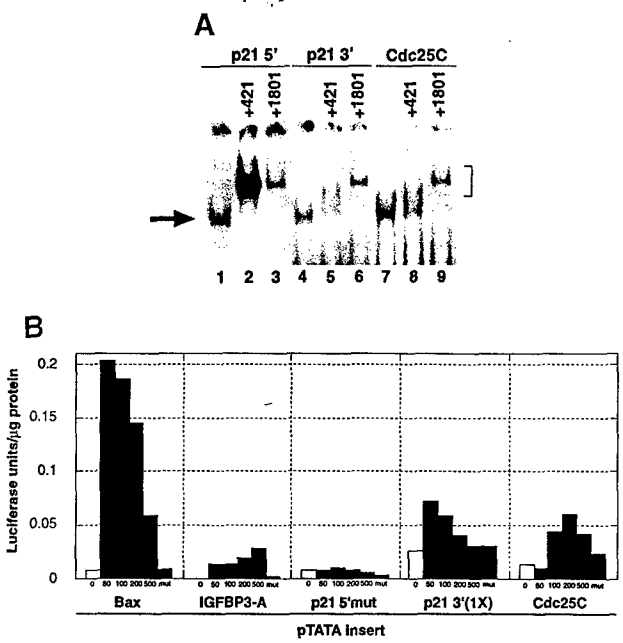

Figure 5. The p53-response element in the promoter of the human *cdc25C* gene has properties similar to the 3′ site. (*A*) An EMSA using as radiolabeled probe the 5′ site (lanes *1–3*), the 3′ site (lanes *4–6*), or the site from the *cdc25C* promoter (lanes *7–9*). The probes had an equivalent specific activity of labeling. Five nanograms of purified p53 was incubated in the absence (lanes *1,4,7*) or presence of either 4 μl of mAb 421 (lanes *2,5,8*) or the presence of 4 μl of mAb 1801 (lanes *3,6,9*). (→) The position of the p53–DNA complex; (]) position of the supershifted p53–DNA–antibody complex. (*B*) Saos-2 cells were transfected as described in Materials and Methods with 2 μg of the indicated reporter constructs in the absence (open bars) or presence of increasing amounts of pCMV–p53wt (50, 100, 200, or 500 ng, shaded bars), or 500 ng of pCMV–p53ala143 (solid bars). Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a total level of plasmid DNA of 2.5 μg. Cells were maintained at 37°C and assayed for luciferase activity and total protein levels as described in Materials and Methods. The indicated values are from a representative experiment that had been performed in duplicate.

To determine whether this p53-binding site from the *cdc25C* promoter can act as a p53-response element in cells, an oligonucleotide containing a single copy of the sequence of this site was inserted adjacent to the adenovirus *E1b* minimal promoter in a luciferase reporter plasmid. This construct was compared to constructs containing two previously characterized p53-response elements, namely one from the human bax promoter, and one of the two intronic sites found in the *IGFBP3* gene, the so-called box A site (Buckbinder et al. 1995; Miyashita and Reed 1995; Friedlander et al. 1996; Ludwig et al. 1996). These reporter constructs were compared to a plasmid containing a single copy of the 3′ site from the *p21* promoter and a plasmid containing a single copy of the 5′ site that contains all four C residues altered. Saos-2 cells were transfected with increasing amounts of the wild-type p53 expression vector in the presence of these various reporter constructs. Wild-type p53 activated reporters containing the Bax, IGFBP3-A, 3′ site, and Cdc25C sites, but not a reporter containing the mutated 5′ site (Fig. 5B). This demonstrates that the site from the *cdc25C* promoter is sufficient to confer p53-dependent transcriptional activation on a heterologous luciferase reporter construct. Thus, mAb 421 differentially affects the binding of p53 to two different genomic binding sites that can mediate p53-dependent transcriptional activation.

Discussion

The binding of the mAb 421 to p53 stimulates the ability of p53 to bind to one set of genomic sites that conform to a previously identified consensus sequence and inhibits its ability to bind to another set of genomic sites that deviate from that consensus. This ability to regulate the sequence selectivity of DNA binding by a transcription factor, even in an in vitro setting, is a novel finding. A provocative unanswered question is whether the inhibition seen in the presence of mAb 421 has a physiological counterpart in the cell such that the sequence-specific binding of p53 to elements such as the 3′ site is regulated. The relevance of the mAb 421 effect will remain an open question until cellular conditions are identified that produce selective inhibition of these variant p53-response elements. Previous studies suggest some possible mechanisms, including regulation by the coactivator p300 and phosphorylation by particular kinases. The coactivator p300 recently has been shown to stimulate the sequence-specific DNA-binding activity of p53 (Gu and Roeder 1997), and the DNA-binding activity of p53 can also be stimulated by phosphorylation by casein ki-

nase II, protein kinase C, and cyclin-dependent kinase (Meek et al. 1990; Takenaka et al. 1995; Wang and Prives 1995). Studies to examine the role of these different kinases, as well as p300, in the regulation of the ability of p53 to interact with elements such as the 3′ site in the *p21* promoter are essential to address these possibilities.

Depending on particular cellular conditions, the tumor suppressor protein p53 has been reported to induce growth arrest in both the G_1 and G_2 phases of the cell cycle, mediate an apoptotic response, or trigger alternatively a differentiation or a senescence pathway (see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997; Sugrue et al. 1997). Because the DNA-binding activity of p53 appears to play a role in each of these physiological responses, the ability of p53 to select among various target genes to elicit a particular cellular response is central to the regulation of its biological function. To date, the identification of a mechanism for the regulation of target gene selectivity by p53 has been elusive. The results presented here, albeit under nonphysiological conditions, suggest one potential mechanism by which such selectivity may be achieved. It will be important to determine whether such a mechanism occurs during any of the various cellular responses to p53 and to identify the target genes that are relevant in each situation.

Materials and methods

Plasmids

The expression plasmids pCMV–$p53^{wt}$ and pCMV–$p53^{Ala143}$, encode the indicated human p53 protein under the control of the CMV promoter. These plasmids were referred originally to as pCMV–SN3 and pCMV–CX3, respectively. The reporter plasmid, pGL3–E1bTATA, was constructed by digesting a synthetic double-stranded oligonucleotide, GCGCGGTACCCTCGAGATGCATGAATTCGCTAGCGAGCTCAGGGTATATAATGAAGCTTGGCC, with *Kpn*I and *Hin*dIII and cloning it into the pGL3–Basic vector (Promega), which had been double-digested with *Kpn*I and *Hin*dIII. The resulting plasmid contains a multiple cloning region with the unique restriction sites, *Kpn*I, *Xho*I, *Nsi*I, *Eco*RI, *Nhe*I, and *Sac*I upstream of the minimal adenovirus *E1b* promoter sequence and the coding region for firefly luciferase.

The following synthetic double-stranded oligonucleotides were digested with *Kpn*I and either *Nhe*I [5′, 3′(1×), 3′(2×), Cdc25C, and Bax] or *Sac*I (5′ mut and IGFBP3-A) and cloned into pGL3–E1bTATA, which had been double-digested with *Kpn*I and either *Nhe*I or *Sac*I to produce the appropriate reporter plasmids: 5′-AATTGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; 3′(1×)–AATTCGGTACCGAAGAAGACTGGGCATGTCTGCTAGCGAATT; 3′(2×)–AATTCGGTACCGAAGAAGACTGGGCATGTCTGAAGAAGACTGGGCATGTCTGCTAGCGAATT; 5′ mut–AATTCGGTACCGAATATATCCCAATATATTGGAGCTCGAATT; Cdc25C–AATTCGGTACCGGGCAAGTCTTACCATTTCCAGAGCAAGCACGCTAGCGAATT; Bax–AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGCTAGCGAATT; and IGFBP3-A–AATTCGGTACCAAACAAGCCACCAACATGCTTTGGAGCTCGAATT.

Transfection of reporter constructs

Saos-2 cells were transfected using the DOTAP liposomal transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. Lysates were prepared, total protein concentration was determined, and luciferase assays were quantitated using a TD-20e Luminometer (Turner).

Purification and quantitation of human p53 protein

Sf9 cells that were infected with recombinant baculovirus were lysed in 20 mM HEPES (pH 7.4) containing 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 50 μM leupeptin, and 50 μg/ml aprotinin (lysis buffer). Nuclei were pelleted by centrifugation at 2300 rpm and then resuspended in lysis buffer containing 500 mM NaCl. Extracts were diluted to 100 mM NaCl, applied to a 0.5-ml Ni-NTA-agarose column (Qiagen) that was equilibrated with 20 mM HEPES containing 100 mM NaCl, and eluted with 200 mM imidazole containing 10 mM HEPES, (pH 7.4) and 5 mM NaCl. Fractions of 0.5-ml were collected, dialyzed against 10 mM HEPES (pH 7.4), 5 mM NaCl, 0.1 mM EDTA, 20% glycerol, and 1 mM DTT, aliquoted, and stored at −70°C.

EMSAs

Complementary single-stranded oligonucleotides were annealed. and ends were filled using the Klenow fragment of DNA polymerase to produce the following double-stranded oligonucleotides: p21 5′-AATTCTCGAGGAACATGTCCCAACATGTTGCTCGAGAATT; p21 3′-AATTCTCGAGGAAGAAGACTGGGCATGTCTTCTACCTCGAGAATT; p21 5′ (mutated)–AATTCTCGAGGAATATATCTTGAATTCTTCCTCGAGAATT; p21 5′ (C^{14} to G)–AATTCTCGAGGAACATGTCCCAAGATGTTGCTCGAGAATT; p21 3′ (G^4 to C)–AATTCTCGAGGAACAAGACTGGGCATGTCTTCTACCTCGAGAATT; and Cdc25C–AATTCTCGAGGGGCAAGTCTTACCATTTCCAGAGCAAGCACCTCGAGATT.

Purified p53 protein, 3 ng of labeled double-stranded oligonucleotide, and hybridoma supernatant where appropriate, were incubated in a total volume of 30 μl of DNA binding buffer containing 20 mM HEPES (pH 7.5), 83 mM NaCl, 0.1 mM EDTA, 12% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.7 mM DTT, 133 μg/ml BSA, and 25 μg/ml poly[d(I–C)] for 30 min at room temperature. Samples were loaded on a native 4% acrylamide gel in 0.5× TBE and electrophoresed at 4°C at 200 V for 2 hr. The gel was dried and exposed to Kodak XAR-5 film using an intensifying screen at −70°C. Bands were quantitated using the Molecular Analyst Phosphorimaging system (Bio-Rad).

Acknowledgments

We thank Bert Vogelstein for the p53 expression plasmids, and Ze'ev Ronai for the recombinant baculovirus expressing His-tagged human p53. Ron Magnusson is thanked for help with the recombinant baculovirus infections. Helpful discussions with Avrom Caplan, Robert Krauss, Jonathan Licht, Leslie Pick, Serafin Piñol-Roma, and, especially, Zhen-Qiang Pan are gratefully acknowledged. Members of the Manfredi laboratory are thanked for their help and support: Yvette Tang, Edward Thornborrow, Michelle Denberg, and Joyce Meng. These studies were supported by grants from the National Cancer Institute (CA69161) and the Breast Cancer Program of the U.S. Army Medical Research and Materiel Command (U.S. AMRMC; DAMD-17-97-1-7336 and DAMD-17-97-1-7337). S.S.C. was supported by a training grant from the Breast Cancer Program of the U.S. AMRMC (DAMD-17-94-J-4111).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Aloni-Grinstein, R., I. Zan-Bar, I. Alboum, N. Goldfinger, and V. Rotter. 1993. Wild type p53 functions as a control protein in the differentiation pathway of the B-cell lineage. *Oncogene* **8:** 3297–3305.

Balagurumoorthy, P., H. Sakamoto, M.S. Lewis, N. Zambrano, G.M. Clore, A.M. Gronenborn, E. Appella, and R.E. Harrington. 1995. Four p53 DNA-binding domain peptides bind natural p53-response elements and bend the DNA. *Proc. Natl. Acad. Sci.* **92:** 8591–8595.

Brugarolas, J., C. Chandrasekaran, J.I. Gordon, D. Beach, T. Jacks, and G.J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377:** 552–557.

Buckbinder, L., R. Talbott, S. Velasco-Miguel, I. Takenaka, B. Faha, B.R. Seizinger, and N. Kley. 1995. Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377:** 646–649.

Cross, S.M., C.A. Sanchez, C.A. Morgan, M.K. Schimke, S. Ramel, R.L. Izerda, W.H. Rashkind, and B.J. Reid. 1995. A p53-dependent mouse spindle checkpoint. *Science* **267:** 1353–1355.

Deng, C., P. Zhang, J.W. Harper, S.J. Elledge, and P. Leder. 1995. Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. *Cell* **82:** 675–684.

El-Deiry, W.S., S.E. Kern, J.A. Pietenpol, K.W. Kinzler, and B. Vogelstein. 1992. Definition of a consensus binding site for p53. *Nature Genet.* **1:** 45–49.

El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817–825.

El-Deiry, W.S., T. Tokino, T. Waldman, J.D. Oliner, V.E. Velculescu, M. Burrell, D.E. Hill, E. Healy, J.L. Rees, S.R. Hamilton, K.W. Kinzler, and B. Vogelstein. 1995. Topological control of $p21^{WAF1/CIP1}$ expression in normal and neoplastic tissues. *Cancer Res.* **55:** 2910–2919.

Friedlander, P., Y. Haupt, C. Prives, and M. Oren. 1996. A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. *Mol. Cell. Biol.* **16:** 4961–4971.

Friedman, P.N., X. Chen, J. Bargonetti, and C. Prives. 1993. The p53 protein is an unusually shaped tetramer that binds directly to DNA. *Proc. Natl. Acad. Sci.* **90:** 3319–3323.

Funk, W.D., D.T. Pak, R.H. Karas, W.E. Wright, and J.W. Shay. 1992. A transcriptionally active DNA-binding site for human p53 protein complexes. *Mol. Cell. Biol.* **12:** 2866–2871.

Gottlieb, T.M. and M. Oren. 1996. p53 in growth control and neoplasia. *Biochim. Biophys. Acta* **1287:** 77–102.

Gu, W. and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90:** 595–606.

Halazonetis, T.D., L.J. Davis, and A.N. Kandil. 1993. Wild-type p53 adopts a "mutant"-like conformation when bound to DNA. *EMBO J.* **12:** 1021–1028.

Hupp, T.R., D.W. Meek, C.A. Midgely, and D.P. Lane. 1992. Regulation of the specific DNA binding function of p53. *Cell* **71:** 875–886.

Hupp, T.R., A. Sparks, and D.P. Lane. 1995. Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell* **83:** 237–245.

Ko, L.J. and C. Prives. 1996. p53: Puzzle and paradigm. *Genes & Dev.* **10:** 1054–1072.

Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88:** 323–331.

Ludwig, R.L., S. Bates, and K.H. Vousden. 1996. Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. *Mol. Cell. Biol.* **16:** 4952–4960.

Macleod, K.F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes & Dev.* **9:** 935–944.

Meek, D.W., S. Simon, U. Kikkawa, and W. Eckhart. 1990. The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. *EMBO J.* **9:** 3253–3260.

Miyashita, T. and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80:** 293–299.

Mundt, M., T. Hupp, M. Fritsche, C. Merkle, S. Hansen, D. Lane, and B. Groner. 1997. Protein interactions at the carboxyl terminus of p53 result in the induction of its in vitro transactivation potential. *Oncogene* **15:** 237–244.

Nagaich, A.K., E. Appella, and R.E. Harrington. 1997a. DNA bending is essential for the site-specific recognition of DNA response elements by the DNA binding domain of the tumor suppressor protein p53. *J. Biol. Chem.* **272:** 14842–14849.

Nagaich, A.K., V.B. Zhurkin, H. Sakamoto, A.A. Gorin, G.M. Clore, A.M. Gronenborn, E. Appella, and R.E. Harrington. 1997b. Architectural accommodation in the complex of four p53 DNA binding domain peptides with the p21/waf1/cip1 DNA response element. *J. Biol. Chem.* **272:** 14830–14841.

Shaulsky, G., N. Goldfinger, A. Peled, and V. Rotter. 1991. Involvement of wild-type p53 in pre-B-cell differentiation in vitro. *Proc. Natl. Acad. Sci.* **88:** 8982–8986.

Soddu, S., G. Blandino, G. Citro, R. Scardigli, G. Piaggio, A. Ferber, B. Calabretta, and A. Sacchi. 1994. Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood* **83:** 2230–2237.

Sugrue, M.M., D.Y. Shin, S.W. Lee, and S.A. Aaronson. 1997. Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proc. Natl. Acad. Sci.* **94:** 9648–9653.

Takenaka, I., F. Morin, B.R. Seizinger, and N. Kley. 1995. Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. *J. Biol. Chem.* **270:** 5405–5411.

Wang, Y. and C. Prives. 1995. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature* **376:** 88–91.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 44, Issue of October 30, pp. 29156–29163, 1998
Printed in U.S.A.

Constitutive Expression of the Cyclin-dependent Kinase Inhibitor p21 Is Transcriptionally Regulated by the Tumor Suppressor Protein p53*

(Received for publication, April 24, 1998, and in revised form, August 7, 1998)

Hsin-yi Tang, Kathy Zhao, Joseph F. Pizzolato, Maxim Fonarev, Jessica C. Langer, and James J. Manfredi‡

From the Derald H. Ruttenberg Cancer Center and the Brookdale Center for Molecular and Developmental Biology, Mount Sinai School of Medicine, New York, New York 10029

The tumor suppressor protein p53 has been implicated in the response of cells to DNA damage. Studies to date have demonstrated a role for p53 in the transcriptional activation of target genes in the cellular response to DNA damage that results in either growth arrest or apoptosis. In contrast, here is demonstrated a role for p53 in regulating the basal level of expression of the cyclin-dependent kinase inhibitor p21 in the absence of treatment with DNA-damaging agents. Wild-type p53-expressing MCF10F cells had detectable levels of p21 mRNA and protein, whereas the p53-negative Saos-2 cells did not. Saos-2 cells were infected with recombinant retrovirus to establish a proliferating pool of cells with a comparable constitutive level of expression of wild-type p53 protein to that seen in untreated MCF10F cells. Restoration of wild-type but not mutant p53 expression recovered a basal level of expression of p21 in these cells. Constitutive expression of luciferase reporter constructs containing the p21 promoter was inhibited by co-transfection with the human MDM2 protein or a dominant-negative p53 protein and was dependent on the presence of p53 response elements in the reporter constructs. Furthermore, p53 in nuclear extracts of untreated cells was capable of binding to DNA in a sequence-specific manner. These results implicate a role for p53 in regulating constitutive levels of expression of p21 and demonstrate that the p53 protein is capable of sequence-specific DNA binding and transcriptional activation in untreated, proliferating cells.

The tumor suppressor protein p53 is a transcription factor that binds to DNA in a sequence-specific manner, has been implicated in the cellular response to DNA damage, and appears to play a role in a variety of cellular responses including growth arrest, apoptosis, differentiation, and senescence (1–4). Studies to date have documented a role for p53 in transcriptional activation of target genes in response to extracellular stimuli including DNA damage leading to a cellular response involving either growth arrest or apoptosis. DNA-damaging agents trigger an increase in p53 expression leading to activation of particular target genes most notably that of the cyclin-dependent kinase inhibitor, p21 (5). Consistent with this, cells that lack p21 expression have an impaired p53-dependent response to DNA damage (6, 7). This transcriptional activation of p21 expression is mediated by the interaction of p53 with two response elements located in the p21 promoter (8).

The DNA binding activity of p53 appears to be regulated by the terminal 30 amino acids of the protein. Phosphorylation by either casein kinase II or protein kinase C, acetylation by p300, and binding by a monoclonal antibody 421, or the bacterial dnaK protein all occur within this region of p53 and will activate the ability of p53 to bind to DNA in a sequence-specific manner *in vitro* (9–15). There have been several reports that the ability of p53 in nuclear extracts to bind to DNA requires the presence of antibody 421, leading to the notion that p53 exists in a latent form prior to DNA damage (10, 12). Consistent with this idea, microinjection of the antibody 421 into cells activates p53-dependent expression from reporter constructs (13, 16). Thus, it has been proposed that in untreated cells, the p53 protein exists in a latent state that is unable to bind to DNA and that the ability of p53 to activate target gene expression is not merely dependent on the increase in protein level but also requires post-translational modification of p53 to convert this latent form into a form that is active for DNA binding (12, 17). This notion is supported by studies demonstrating that p53 becomes phosphorylated at particular sites after treatment of cells with DNA-damaging agents (18, 19).

Prior to the cloning of the gene, it was noted that p21 was absent from cyclin/cyclin-dependent kinase complexes in cells lacking functional p53 (20). Other studies have noted that the level of p21 mRNA was much lower in fibroblasts and keratinocytes derived from mice containing a homozygous deletion of p53 as compared with the corresponding cells from mice expressing wild-type p53 (21–24). This suggests that p53 may play a role in the level of p21 expression in untreated, proliferating cells. The experiments presented here tested this idea directly and demonstrate that constitutive expression of the p21 protein in untreated cells is, indeed, dependent on p53 and thus implicate a role for p53 not only in the increased expression of p21 in response to DNA damage leading to either growth arrest or apoptosis but also in the basal level of expression of p21 in normally proliferating cells.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid p21P contains 2.5-kb[1] of the human *p21* promoter inserted upstream of a firefly luciferase reporter gene in the

* This work was supported by Public Health Service Grant CA-69161 from the National Cancer Institute and Grants DAMD-17-97-1-7336 and DAMD-17-97-1-7337 from the Breast Cancer Program of the U. S. Army Medical Research and Materiel Command. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Derald H. Ruttenberg Cancer Center, Box 1130, Mount Sinai School of Medicine, New York, NY 10029. Tel.: 212-824-8110; Fax: 212-849-2446; E-mail: jmanfredi@smtplink.mssm.edu.

[1] The abbreviations used are: kb, kilobase pair(s); CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HMBA, *N,N'*-hexamethylene-bisacetamide.

vector pGL2 (Promega). The plasmid p21D2.1 has 2.1 kb at the 5′ end of the promoter sequence removed and lacks the two p53 response elements of the p21 promoter (25). The plasmid pRL-SV40 contains the SV40 enhancer and early promoter upstream of a Renilla luciferase reporter gene (Promega). The plasmid pCMV-hdm2 encodes the human MDM2 protein under control of the cytomegalovirus (CMV) promoter and the plasmid pCMV-p53Ala-143 encodes the tumor-derived mutant human p53 protein containing a missense mutation of valine to alanine at residue 143 (26).

Antibodies and Cells—Saos-2 and WI38 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MCF7 cells were maintained in RPMI medium containing 10% heat-inactivated FBS and 5 μg/ml insulin. MCF10F cells were grown in 50% DMEM and 50% Ham's F12 medium containing 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 500 ng/ml hydrocortisone. PA12-p53BN and PA12-p53EN are cell lines that produce recombinant retrovirus encoding human wild-type p53 or the mutant $p53^{His-273}$, respectively (27). These cell lines were grown in 10% FBS in DMEM containing high glucose and 400 μg/ml G418 sulfate. The hybridoma cell line producing the mouse monoclonal antibody 1801 was grown in DMEM containing 10% FBS. Hybridoma cell lines expressing the mouse monoclonal antibodies 421 and 419 were grown in 50% DMEM and 50% Fischer's medium containing 10% FBS. Monoclonal antibody 1801 specifically reacts with human p53 (28), 421 recognizes p53 from a variety of species, and 419 recognizes an epitope on the SV40 large T antigen (29). All cell lines were grown at 37 °C in a humid incubator containing 5% CO_2. Antibody against $p21^{WAF1, CIP1}$ was obtained commercially (Ab-1/clone EA10, Calbiochem). For treatment with ultraviolet light, the medium was removed, and the cells were exposed to ultraviolet light using a UV Stratalinker (Stratagene).

Northern Analysis—Total RNA was extracted from 5×10^6 cells using RNAzol (Tel-test), and Northern blot analysis was performed following conventional procedures, using a 2.1-kb full-length human p21 cDNA or human glyceraldehyde-3-phosphate dehydrogenase cDNA (Ambion) as probes.

Immunoblotting—Cells were lysed in a buffer containing 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate (SDS), 50 mM NaCl, 25 mM Tris-HCl, pH 7.5, and the protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), aprotinin (50 μg/ml), and leupeptin (50 μg/ml) for 10 min on ice. Lysates were spun at 15,000 rpm for 10 min, and the supernatant was saved. Protein levels were determined by the bicinchoninic acid protein assay (Pierce). Appropriate amounts of total cellular protein were loaded on 10% SDS-polyacrylamide gels and electrophoresed at 150 V constant voltage for 3 h. Samples were transferred to nitrocellulose paper and probed with the appropriate antibody. Second antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG, and the signal was detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

Establishment of Retrovirally Infected Cells Expressing Ectopic p53—The retrovirus-producing cell lines PA12-BN and PA12-EN were grown to 75% confluence and fed with fresh DMEM containing 10% FBS. After incubation at 37 °C for 16 h, the supernatant was harvested and filtered through a 0.2-μm filter. Old medium was removed from a subconfluent 60-mm dish of Saos-2 cells and replaced with 1 ml of filtered supernatant containing 8 μg/ml Polybrene. Dishes were rocked for 2 h at 37 °C in a humid incubator containing 5% CO_2 and then 3 ml of DMEM containing 10% FBS was added to the dish, and it was further incubated for 48 h. The cells were then trypsinized and replated in a 100-mm dish using DMEM containing 10% FBS and 400 μg/ml G418 sulfate. Cells were fed every 3 days with this same medium. After 2 weeks, the resulting drug-resistant colonies were pooled and passaged.

Incorporation of Bromodeoxyuridine—For detecting replicative DNA synthesis, cells were incubated with 10 μM bromodeoxyuridine for 30 min prior to fixation. The proportion of cells actively synthesizing DNA was quantitated by anti-bromodeoxyuridine immunoanalysis, and the total DNA content was analyzed by staining with propidium iodide as described previously (30). Cells were fixed with 70% ethanol for at least 2 h, resuspended in the following solutions in order: 0.25% paraformaldehyde in phosphate-buffered saline (PBS), 0.5 mg/ml ribonuclease A in PBS, 0.5% Triton X-100 in 0.1 N HCl, and finally distilled water. Samples were then heated at 97 °C for 10 min, immediately placed on ice for additional 10 min, and washed with 0.5% Tween 20 in PBS. The incorporation of bromodeoxyuridine was detected by monoclonal anti-bromodeoxyuridine antibody conjugated to fluorescein isothiocyanate (Becton Dickinson). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson).

Transfection of Reporter Constructs—MCF7, MCF10F, or Saos-2 cells were transfected using the *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium salts liposomal transfection reagent (DOTAP, Boehringer Mannheim). One confluent 100-mm dish of cells was split into three 6-well dishes and incubated for 24 h. Cells were fed with complete medium containing serum and incubated for an additional 3 h. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium salts/DNA mixtures containing 2 μg of the relevant reporter plasmid plus 50 ng of the p53 expression plasmid or an equal amount of an empty vector plasmid were prepared according to the manufacturer's instructions and incubated at room temperature for 15 min. Serum-free medium was then added to the mixtures and used to replace the media in the wells. The dishes were incubated at 37 °C for 3 h, after which the transfection mix was removed and replaced with complete medium containing serum. After 48 h, the 6-well plates were placed on ice and washed once with PBS. The cells were then lysed by scraping into 120 μl of Reporter Buffer (Promega Luciferase Assay System), and samples were spun for 1 min at 14,000 rpm at 4 °C. Total protein concentration was determined using a commercially available assay (Bio-Rad). 40 μl of each sample was warmed to room temperature and mixed with luciferase assay substrate that was reconstituted with Luciferase Assay Buffer (Promega). Light emission was determined in a TD-20e luminometer (Turner).

Preparation of Nuclear and Cytosolic Extracts—Nuclear and cytosolic extracts were performed as described by Graeber *et al.* (31). Cells were homogenized in 10 mM Tris-HCl, pH 7.4, containing 10 mM NaCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 100 μM Na_3VO_4 and spun at 10,000 rpm for 30 s. The supernatant was saved as the cytosolic extract. The pellet was repacked by spinning at 14,000 rpm for 1 min and then nuclei were suspended in a nuclear extraction buffer (20 mM Hepes, pH 7.5, containing 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin), incubated at 4 °C for 1 h, and spun at 14,000 rpm for 10 min. This supernatant was saved as the nuclear extract. Lactate dehydrogenase activity was assayed according to Ramirez *et al.* (32), and histone levels were determined by immunoblotting using an anti-histone antibody that reacts with an epitope that is present on all five histone proteins (H11-4, Boehringer Mannheim). Such assays showed less than 10% cross-contamination between cytosolic and nuclear extracts.

Electrophoretic Mobility Shift Assays—The specific probe that was used for binding, TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC, contains the high affinity binding sequence identified by Halazonetis *et al.* (11) named by them BC or BB.9. In the competition experiments, the nonspecific oligonucleotide (referred to as Sens-1), TCGAAGAAGACGTGCAGGGACCC, was used. Complementary single-stranded oligonucleotides were annealed by incubation at 95 °C for 4 min, 65 °C for 10 min, and then gradually brought to room temperature. Ends were filled using the Klenow fragment of DNA polymerase to produce a labeled double-stranded oligonucleotide. Appropriate amounts of extracts (1–7 μl) were mixed with 1 ng of labeled double-stranded oligonucleotide in a total reaction mixture of 30 μl containing 6 μl of 5× electrophoretic mobility shift assay buffer (100 mM Hepes, pH 7.9, 0.5 mM EDTA, 50% glycerol, 10 mM $MgCl_2$), 1.5 μl of 40 mM spermidine, 1.5 μl of 10 mM dithiothreitol, 1 μl of 500 μg/ml double-stranded poly(dI/dC), and 5–13 μl of water with a final salt concentration of 85 mM. The amount of total protein per reaction was normalized, and the reactions were carried out at room temperature for 30 min. For antibody supershift analysis, 2 μl of the appropriate undiluted hybridoma supernatant was added. His-tagged human p53 was produced by infection of insect cells with a recombinant baculovirus and purified by nickel-agarose chromatography and used as a positive control (52). Samples were electrophoretically separated on a native 4% polyacrylamide gel at 4 °C at 200 V for 2 h. After drying, gels were exposed to Kodak XAR film at −70 °C with an intensifying screen.

RESULTS

MCF10F Cells Express Detectable Levels of p21 mRNA and Protein, Whereas p53-Negative Saos-2 Cells Do Not—Previous studies have noted that either fibroblasts or keratinocytes from mice that were homozygously deleted for p53 expressed lower basal levels of p21 mRNA as compared with fibroblasts or keratinocytes from mice expressing both alleles of the wild-type p53 gene (21–24). To characterize further a role for p53 in the basal level of expression of p21, the p53-negative cell line

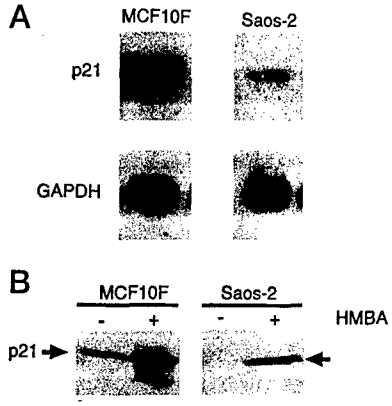

FIG. 1. **MCF10F cells express detectable levels of p21 mRNA and protein, whereas p53-negative Saos-2 cells do not.** *A*, RNA was extracted from wild-type p53-expressing MCF10F and p53-negative Saos-2 cells, and Northern analysis was performed as described under "Experimental Procedures." Blots were probed with either a cDNA for human p21 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as indicated. *B*, MCF10F or Saos-2 cells were left untreated or treated with 10 mM HMBA for 16 h. Whole cell extracts were prepared and subjected to electrophoresis and subsequent immunoblotting using an anti-p21 antibody as described under "Experimental Procedures."

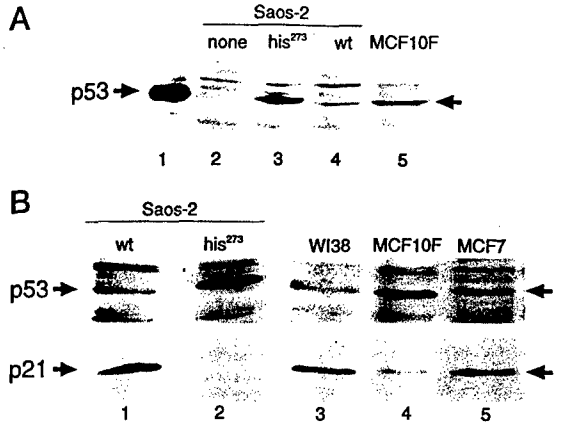

FIG. 2. **Retroviral infection of Saos-2 cells restores expression of p53 and p21.** *A*, Saos-2 cells were infected with a recombinant retrovirus expressing either wild-type p53 or the mutant $p53^{His-273}$. Selection was performed in G418 sulfate, and drug-resistant pools of cells were obtained. Equivalent amounts of total cellular extract of parent Saos-2 cells (*lane 2*), the His^{273}-expressing Saos-2 cells (*lane 3*), the wild-type (*wt*) p53-expressing Saos-2 cells (*lane 4*), or MCF10F cells (*lane 5*) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with an anti-p53 antibody. Purified human p53 is shown in *lane 1*. *B*, equivalent amounts of total cellular extract of Saos-2 cells expressing either wild-type p53 (*lane 1*) or the His^{273} mutant p53 (*lane 2*) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either an anti-p53 or anti-p21 antibody.

Saos-2 was compared with the wild-type p53-expressing cell line MCF10F. Total RNA was extracted from each cell line, and Northern analysis was performed. The p53-negative Saos-2 cell line expressed low levels of p21 mRNA as compared with the wild-type p53-expressing MCF10F cells (Fig. 1*A*). Total cellular extracts of each cell line were subjected to SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with an anti-p21-specific antibody (Fig. 1*B*). MCF10F cells expressed a detectable level of p21, whereas the level of p21 expression in Saos-2 cells was undetectable. To confirm that Saos-2 cells retained the ability to synthesize p21, both MCF10F and Saos-2 cells were treated with 10 mM *N,N'*-hexamethylenebisacetamide (HMBA). HMBA is a non-retinoid, differentiating agent that has previously been shown to induce p21 expression in a p53-independent manner (24). Treatment of Saos-2 cells with HMBA induced expression of p21 demonstrating that Saos-2 cells retained the ability to synthesize p21. Thus, both the level of protein and messenger RNA for p21 were much higher in the p53-expressing MCF10F cells than in the p53-negative Saos-2 cells.

Retroviral Infection of Saos-2 Cells Restores Expression of p53 and p21—Previous studies have shown that restoration of wild-type p53 expression through transfection of a suitable expression plasmid did not allow for establishment of stable cell lines expressing wild-type p53 (33–37). This was presumably due to the fact that plasmid transfection results in a high level of expression of p53 which is incompatible with cell proliferation. Chen *et al.* (27) utilized recombinant retroviral infection to restore a level of wild-type p53 expression in Saos-2 cells that was comparable to that seen in normal cells and that was compatible with continued proliferation of these cells. To that end, Saos-2 cells were infected with recombinant retroviruses expressing either wild-type human p53 or the mutant human $p53^{His-273}$, and pools of G418 sulfate-resistant cells were established. Immunoblotting of whole cell extracts from these drug-resistant pools demonstrated that both wild-type (Fig. 2*A*, *lane 4*) and mutant (Fig. 2*A*, *lane 3*) p53 expression could be detected in comparison to the parent cells which are p53-negative (Fig. 2*A*, *lane 2*). Furthermore, the pool of Saos-2 cells expressing wild-type p53 expressed a level that is comparable to the endogenous p53 level in MCF10F cells (Fig. 2*A*, *lane 5*). Consistent with previous observations, this level of expression of wild-type p53 that was obtained using recombinant retroviral infection was sufficiently low to allow the cells to continue to grow (Table I). These drug-resistant pools were labeled with bromodeoxyuridine and subjected to flow cytometric analysis to demonstrate that they were actively incorporating DNA. Indeed, the pools expressing wild-type p53 had a similar percentage of bromodeoxyuridine-positive cells as the parent cell line, the pool expressing mutant p53, or the wild-type p53 expressing cell lines WI38, MCF10F, or MCF7 (Table I). These pools were then examined for the level of p21 expression. Immunoblotting of whole cell extracts demonstrated that Saos-2 cells expressing wild-type but not mutant p53 expressed a level of p21 that was comparable to that of WI38 or MCF7 cells and, in fact, was greater than that seen with MCF10F cells (Fig. 2*B*). Thus, restoration of expression of wild-type p53 in a p53-negative cell line also restored a basal level of expression of p21.

Constitutive Expression of Luciferase Reporter Constructs Containing the p21 Promoter Is p53-dependent—The observation that reintroduction of p53 expression in Saos-2 cells restored a basal level of p21 expression (Fig. 2) suggests that in the absence of DNA damage, p53 regulates expression of p21. To test directly this notion and to confirm that such regulation is at the level of transcription, wild-type p53-expressing MCF7 cells were transfected with a luciferase reporter construct containing 2.4 kb of the human p21 promoter. To determine whether the basal level of expression that is observed was p53-dependent, an expression plasmid for the human MDM2 protein was co-transfected with the reporter. Mdm2 binds to p53 and inhibits its transcriptional activity, apparently by targeting the p53 protein for degradation (19, 38–41). Transfection of MCF7 cells with a luciferase reporter construct under control of the p21 promoter, p21P, confirmed a basal level of activation of the p21 promoter (Figs. 3 and 4*A*). Co-transfection of an expression plasmid encoding human MDM2 protein caused repression of that basal level of expression (Fig. 3 and 4*A*). Deletion of the p53-binding sites from this reporter (p21P 2.1) resulted in a complete loss of basal luciferase activity (Fig. 3). In contrast, co-transfection of the plasmid encoding Mdm2

TABLE I
Incorporation of bromodeoxyuridine into the DNA of various cell lines

Cell line	Bromodeoxyuridine positive cells[a]
	%
WI38	12
MCF10F	22
MCF7	27
Saos-2	24
Saos-2 (wt)[b]	23
Saos-2 (His^{273})[b]	24

[a] Cells were labeled with 1 μM bromodeoxyuridine for 30 min, fixed, and processed for flow cytometric analysis as described under "Experimental Procedures."

[b] Pools of G418 sulfate-resistant Saos-2 cells that had been infected with recombinant retrovirus expressing either wild-type (wt) or mutant (His^{273}) p53 proteins.

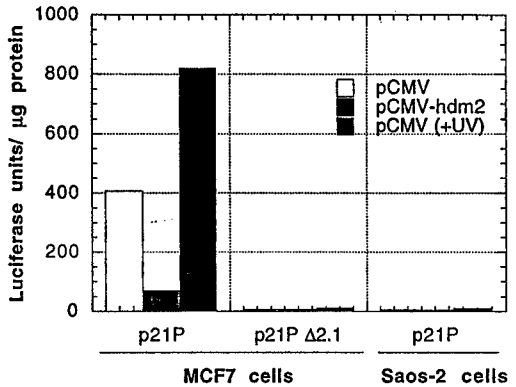

FIG. 3. **Deletion of the p53 response elements results in loss of basal expression of a luciferase reporter containing the p21 promoter.** MCF7 cells or Saos-2 cells were transfected as described under "Experimental Procedures" with 2 μg of p21P or p21P 2.1 and either 50 ng of pCMV or pCMV-hdm2 as indicated. 18 h prior to lysis, a set of wells containing only pCMV were treated with 50 J/m^2 of ultraviolet light, indicated by *+UV*. 48 h after transfection, cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The indicated values are from a representative experiment that had been performed in duplicate.

into the p53-negative Saos-2 cells had no effect on the low level of luciferase activity seen in these cells from the same reporter construct (Figs. 3 and 4*C*). Treatment of MCF7 cells with ultraviolet light induced expression of the full-length p21 promoter construct but not the construct that lacks the p53-binding sites (Fig. 3). Furthermore, treatment of Saos-2 cells with ultraviolet light had no effect on the expression of luciferase from the full-length p21 promoter reporter construct (Fig. 3). These latter results are consistent with the fact that MCF7 cells express a functional wild-type p53 protein (42–44).[2]

The ability of the Mdm2 protein to repress basal expression from a luciferase reporter containing the full-length p21 promoter was confirmed in the wild-type p53-expressing cell line MCF10F (Fig. 4*B*). Similar to MCF7 cells (Fig. 4*A*) and in contrast to the p53-negative Saos-2 cells (Fig. 4*C*), co-transfection of the expression plasmid for Mdm2 protein inhibited the constitutive level of expression that is seen with the reporter p21P. The apparently low level of basal expression seen in Saos-2 cells further strengthens the notion that the basal level of expression is p53-dependent. To confirm that this is indeed the case and not a reflection of different transfection efficiencies by the various cell lines, MCF7, MCF10F, and Saos-2 cells were co-transfected with p21P and an additional reporter that contains Renilla luciferase under the control of the SV40 enhancer and early promoter. This latter reporter construct was used to normalize for transfection efficiency. The results of this analysis demonstrated that, indeed, both MCF7 and MCF10F have a much higher basal level of expression of p21P than the p53-negative Saos-2 cells (Fig. 4*D*).

To provide further evidence that the basal expression that is seen upon transfection of MCF7 cells with p21P is p53-dependent, MCF7 and Saos-2 cells were co-transfected with p21P and increasing amounts of an expression plasmid encoding a dominant-negative mutant $p53^{Ala\text{-}143}$ (45). Increasing amounts of the $p53^{Ala\text{-}143}$ expression plasmid repressed the basal level of expression of p21P in MCF7 cells but not in Saos-2 cells (Fig. 5). Thus, co-transfection of either a dominant-negative p53 or the human MDM2 protein, both of which are capable of inhibiting the endogenous wild-type p53, caused repression of the basal level of expression from the reporter construct containing the p21 promoter. In contrast, co-transfection of the dominant-negative p53 or the human MDM2 protein into the p53-negative Saos-2 cells had no effect on the low level of luciferase activity seen in these cells from the same reporter construct.

p53 in Nuclear Extracts of Untreated Cells Is Capable of Binding to DNA in a Sequence-specific Manner—The data presented thus far implicate a role for endogenous p53 in transcriptional activation of the p21 promoter in untreated proliferating cells. If this is the case, then this endogenous p53 should be capable of binding to DNA. To examine this, electrophoretic mobility shift assays utilized nuclear extracts from three different wild-type p53-expressing cell lines, WI38, MCF10F, and MCF7 to demonstrate that, indeed, the endogenous p53 was capable of binding to DNA prior to DNA damage. All three cell lines were either untreated or treated with 50 J/m^2 of ultraviolet light and then were fractionated into nuclear and cytosolic extracts. Immunoblotting for p53 demonstrated that prior to DNA damage, WI38 and MCF10F cells express a p53 that was primarily localized to the nucleus (Fig. 6, *lanes 1* and *2*, and *5* and *6*), whereas the p53 in untreated MCF7 cells was present primarily in the cytoplasm with a low level detectable in the nuclear fraction (Fig. 6, *lanes 9* and *10*). After treatment with ultraviolet light, the p53 levels increased substantially in all three cell lines (Fig. 6, *lanes 3–4, 7* and *8*, and *11–12*). Extracts were assayed for a cytoplasmic marker, lactose dehydrogenase,[3] as described under "Experimental Procedures" and were immunoblotted for a nuclear marker, histone H1 (Fig. 6, *lower panel*). It is estimated that there was less than 10% cross-contamination between the cytoplasmic and nuclear extracts using these markers.

Nuclear and cytoplasmic extracts from untreated and UV-treated MCF cells were normalized for level of p53 protein and used in an electrophoretic mobility shift assay using a consensus p53-binding site as radiolabeled probe (Fig. 7). Both the nuclear and cytoplasmic extracts from UV-treated cells demonstrate a shifted complex with a similar mobility as that of purified p53 (Fig. 7, *lanes 11* and *14*). The p53-specific antibody 1801 efficiently supershifted this complex, whereas the nonspecific antibody 419 did not (Fig. 7, *lanes 12–13* and *15–16*). The extracts from untreated cells contained a shifted complex with a similar mobility as purified p53 (Fig. 7, *lanes 5* and *8*). Incubation with the p53-specific antibody 1801 produced a slower migrating complex but did not substantially affect the original protein-DNA complex (Fig. 7, *lanes 6* and 9). The nonspecific antibody 419 had no such effect (Fig. 7, *lanes 7* and *10*). This result suggests that there is p53 in these extracts which is capable of binding to DNA, but there is also an addi-

[2] H.-Y. Tang, K. Zhao, J. Langer, S. Waxman, and J. J. Manfredi, submitted for publication.

[3] H.-Y. Tang and J. J. Manfredi, unpublished observations.

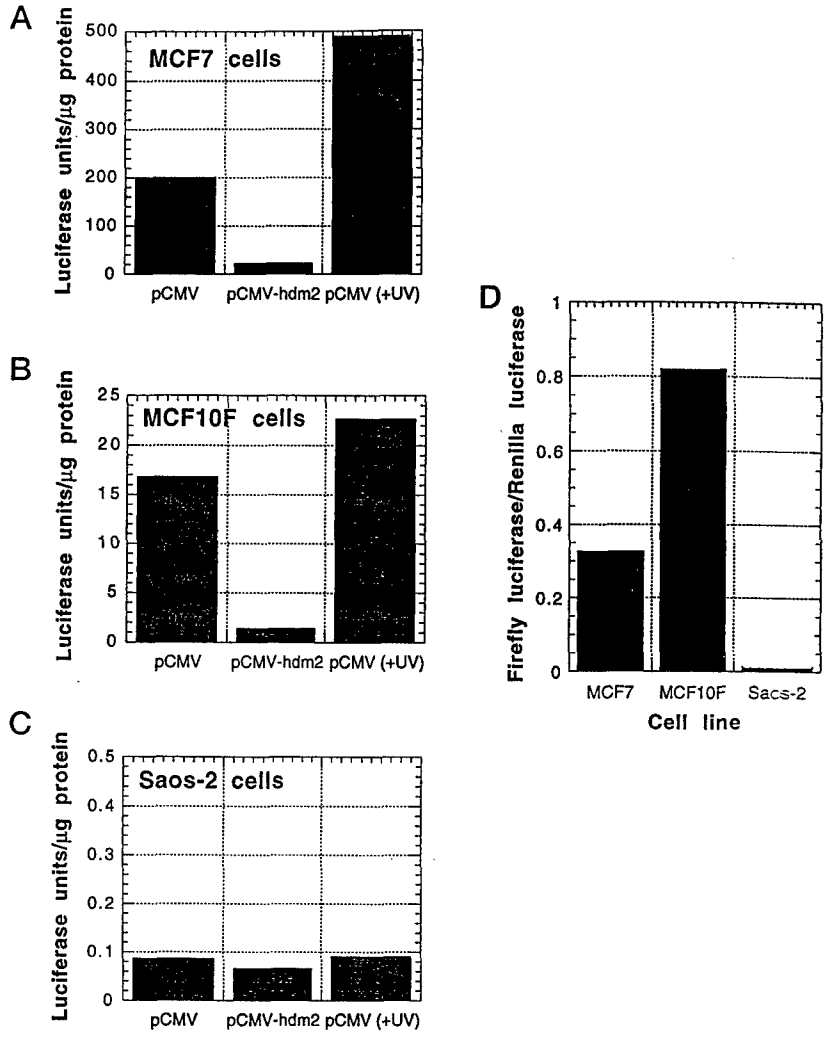

FIG. 4. **Ectopic expression of the human MDM2 protein represses basal expression of a luciferase reporter containing the p21 promoter.** MCF7 cells (*A*), MCF10F cells (*B*), or Saos-2 cells (*C*) were transfected as described under "Experimental Procedures" with 2 μg of p21P and either 50 ng of pCMV or pCMV-hdm2 as indicated. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." 18 h prior to lysis, a set of wells containing only pCMV were treated with 50 J/m^2 of ultraviolet light, indicated by *+UV*. The *bars* represent the average of three independent experiments that had been performed in duplicate. *D*, MCF7 cells, MCF10F cells, or Saos-2 cells were transfected as described under "Experimental Procedures" with 2 μg of p21P and 50 ng of pRL-SV40. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The *bars* represent the average of two independent experiments that had been performed in duplicate.

tional DNA-binding protein that is distinct from p53 which produces a shifted complex of similar mobility as purified p53. To test this, nuclear extracts of untreated MCF7 cells were immunoprecipitated with an anti-p53 antibody to clear all detectable p53 protein from the extract as determined by immunoblotting.[4] This extract was compared in an electrophoretic mobility shift assay with a comparable extract that had been immunoprecipitated with the nonspecific antibody 419 as a control as well as a nuclear extract for UV-treated cells that had similarly been immunoprecipitated with 419 (Fig. 8*A*). Incubation of the p53-specific antibodies 1801 or 421 with untreated nuclear extracts resulted in the detection of slow migrating DNA-protein complexes that were not present in the absence of antibody (Fig. 8*A*, *lanes 2* and *3*). These slower migrating complexes were not seen in an extract that had been cleared of p53 by immunoprecipitation but were present in extract that had been immunoprecipitated with a nonspecific antibody (Fig. 8*A*, *lanes 5* and *6* and *8* and *9*). Clearing of p53 from the extract had no effect on the protein-DNA complex that migrated to a similar mobility as the p53-DNA complex, confirming that there is a DNA-binding protein in the extract which is distinct from p53. Extracts from UV-treated cells were used to identify the p53-DNA complex that was confirmed by its ability to be efficiently supershifted by both 1801 and 421 (Fig. 8*A*, *lanes 11* and *12*). To determine that the binding that was seen was sequence-specific, competition experiments were performed (Fig. 8*B*). Nuclear extract from untreated MCF7 cells was used in an electrophoretic mobility shift assay in the presence of increasing amounts of either specific probe, BB.9, or a nonspecific probe, Sens-1. Since it was difficult to detect the p53-DNA complex in the absence of antibody, the competition was also performed in the presence of the p53-specific antibody 1801. Increasing amounts of unlabeled BB.9 (Fig. 8*B*, *lanes 9–11*) competed well for the binding to 1801-supershifted complexes, whereas increasing amounts of Sens-1 (Fig. 8*B*, *lanes 12–14*) did not. The faster migrating complex that did not appear to contain p53 was similarly competed suggested that the binding of this protein is also sequence-specific (Fig. 8*B*).

The untreated MCF7 cells used in these experiments expressed an endogenous p53 that is localized primarily in the cytoplasm (Fig. 6). The DNA binding results were subsequently confirmed in WI38 and MCF10F cells in which the p53 is primarily nuclear prior to DNA damage (Fig. 6). Nuclear and cytoplasmic extracts from untreated and UV-treated WI38 and MCF10F cells were normalized for level of p53 protein and used in similar electrophoretic mobility shift assays (Fig. 9). Both the nuclear and cytoplasmic extracts from UV-treated cells from both cell lines demonstrated a shifted complex with a similar mobility as that of purified p53 (Fig. 9, *A* and *B*, *lanes 11* and *14*). The p53-specific antibody 1801 efficiently supershifted this complex, whereas the nonspecific antibody 419 did not (Fig. 9, *A* and *B*, *lanes 12* and *13*, and *15* and *16*). As seen with extracts from MCF7 cells, the extracts from untreated WI38 or MCF10F cells contained a shifted complex with a similar mobility as purified p53 (Fig. 9*A*, *lane 5*, and 9*B*, *lanes 5* and *8*). Incubation with the p53-specific antibody 1801 pro-

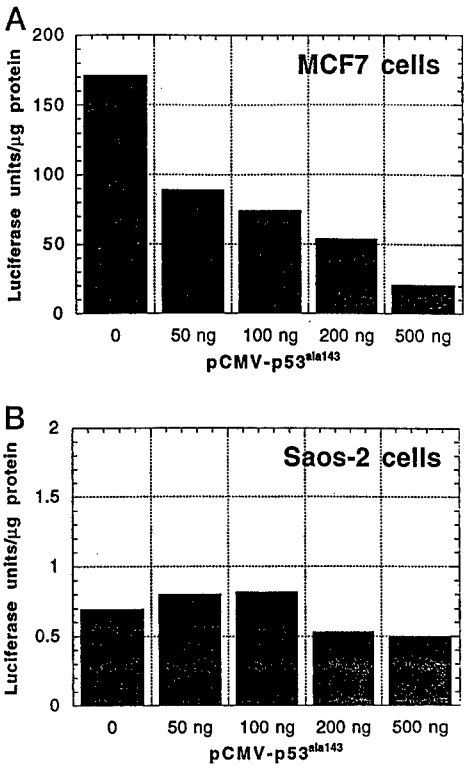

FIG. 5. **Ectopic expression of the mutant human p53$^{Ala-143}$ protein represses basal expression of a luciferase reporter containing the p21 promoter in a dose-dependent manner.** MCF7 cells (*A*) or Saos-2 cells (*B*) were transfected as described under "Experimental Procedures" with 2 μg of p21P alone or in the presence of 50, 100, 200, or 500 ng of pCMV-p53$^{Ala-143}$ as indicated. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The indicated values are from a representative experiment that had been performed in duplicate.

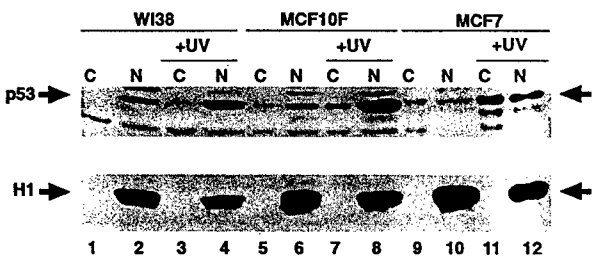

FIG. 6. **Biochemical fractionation demonstrates that nuclear p53 levels increase upon UV treatment in WI38, MCF10F, and MCF7 cells.** WI38, MCF10F, or MCF7 cells were untreated or treated with 50 J/m^2 of ultraviolet light (+*UV*) and then incubated at 37 °C for 20 h prior to fractionation into cytosolic (*C*) or nuclear extracts (*N*) as described under "Experimental Procedures." Samples were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the anti-p53 monoclonal antibody 1801 or a polyclonal antibody directed against histone H1 as indicated. The protein level for the cytosolic samples ranged from 60 to 120 μg. For each cell line, the cytosolic and nuclear samples were normalized so that the loaded samples were obtained from an equivalent number of cells.

duced a slower migrating complex but did not substantially affect the original protein-DNA complex (Fig. 9, *A* and *B*, *lanes 6* and *9*). The nonspecific antibody 419 had no such effect (Fig. 9, *A* and *B*, *lanes 7* and *10*). These results are consistent with those seen with extracts of MCF7 cells demonstrating that there is p53 in these extracts that is capable of binding to DNA but there is also an additional DNA-binding protein that is distinct from p53 that produces a shifted protein-DNA complex of a similar mobility as p53. The presence of a slower migrating shifted complex that was induced by the p53-specific antibody

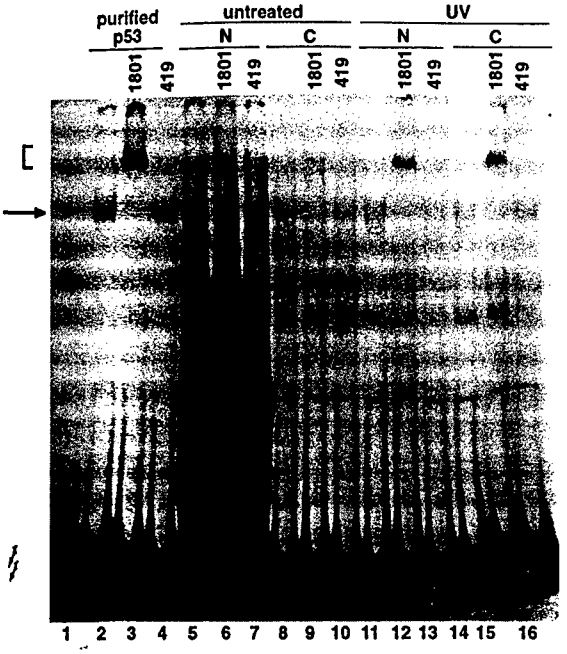

FIG. 7. **p53 in nuclear extracts of untreated MCF7 cells binds to DNA in an electrophoretic mobility shift assay.** Nuclear and cytosolic extracts were prepared from MCF7 cells that were untreated (*lanes 5–10*) or 24 h after treatment with 50 J/m^2 of ultraviolet light (*lanes 11–16*). Electrophoretic mobility shift assay was performed as described under "Experimental Procedures." 1 ng of radiolabeled probe (BB.9) was incubated in the absence of protein (*lane 1*), or in the presence of 0.5 μg of human purified human p53 (*lanes 2–4*) or the appropriate amount of nuclear (*lanes 5–7* and *11–13*) or cytosolic (*lanes 8–10* and *14–16*) extracts as indicated. The level of p53 loaded in cellular extracts was normalized by adjusting the total protein. Incubations were performed either with no addition (*lanes 1, 2, 5, 8, 10*, and *14*) or in the presence of either 1801 (anti-p53 antibody, *lanes 3, 6, 9, 12*, and *15*) or 419 (anti-SV40 large T antigen, *lanes 4, 7, 10, 13*, and *16*). The *arrow* indicates the position of the p53-DNA complex, and the *bracket* indicates the position of the supershifted p53-DNA-antibody complex.

1801 but not the nonspecific antibody 419 in untreated extracts from all three cell lines is consistent with the notion that the endogenous p53 in these cells is capable of binding to DNA in untreated, proliferating cells.

DISCUSSION

Previous studies have suggested that p53 exists in a latent or inactive form in untreated cells and that upon DNA damage not only does the p53 level increase but the p53 itself is modified in some way to activate it for DNA binding and transcriptional activation. Reintroduction of p53 into the p53-negative cell line Saos-2 restored a constitutive level of expression of the cyclin-dependent kinase inhibitor p21 (Fig. 2). A similar result has been reported upon similar retroviral infection of a p53-negative peripheral neuroepithelioma cell line (46). These results suggest that the endogenous p53 in untreated, proliferating cells may be capable of transcriptionally regulating p21 expression. Indeed, experiments involving transfection of wild-type p53-expressing MCF7 cells with a luciferase reporter construct containing the p21 promoter have confirmed that this is the case (Figs. 3–5). A reporter containing the full-length p21 promoter but not a promoter construct in which the p53 response elements have been deleted demonstrated a basal level of expression in MCF7 or MCF10F cells but not in p53-negative Saos-2 cells (Fig. 4). This basal level of expression was inhibited by coexpression of either a dominant-negative p53 or the human MDM2 protein (Figs. 3–5). These results imply that the endogenous p53 in MCF7 or MCF10F cells is capable of binding to DNA. This was directly tested through the use of electro-

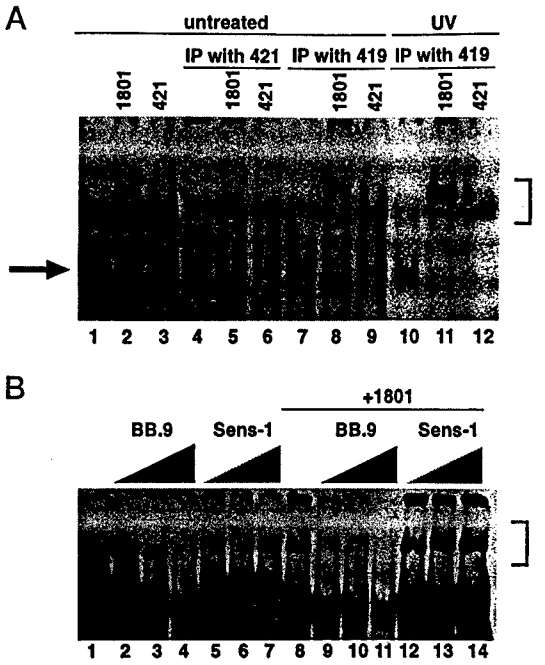

FIG. 8. **p53 in nuclear extracts of untreated MCF7 cells binds to DNA in a sequence-specific manner.** *A*, nuclear extracts of MCF7 cells that were untreated (*lanes 1–9*) or treated with 50 J/m^2 ultraviolet light (UV, *lanes 10–12*) were used directly (*lanes 1–3*) or immunoprecipitated with either an anti-p53 antibody 421 (*lanes 4–6*) or an anti-SV40 large T antigen antibody 419 (*lanes 7–12*). The resulting supernatants were used in an electrophoretic mobility shift assay using 1 ng of radiolabeled probe (BB.9). Incubations were performed either in the absence (*lanes 1, 4, 7,* and *10*) or presence of 1801 (anti-p53 antibody, *lanes 2, 5, 8,* and *11*) or presence of 421 (anti-p534 antibody, *lanes 3, 6, 9,* and *12*). The *arrow* to the *left* indicates the position of the p53-DNA complex, and the *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex. *B*, 1 ng of radiolabeled probe (BB.9) was incubated with an appropriate amount of nuclear extract alone (*lanes 1* and *8*), or in the presence of increasing amounts of unlabeled BB.9 oligonucleotide (*lanes 2–4* and *9–11*) or increasing amounts of a nonspecific oligonucleotide, Sens-1 (*lanes 5–7* and *12–14*). Incubations were performed either in the absence (*lanes 1–7*) or presence of 1801 (anti-p53 antibody, *lanes 8–14*). The *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex.

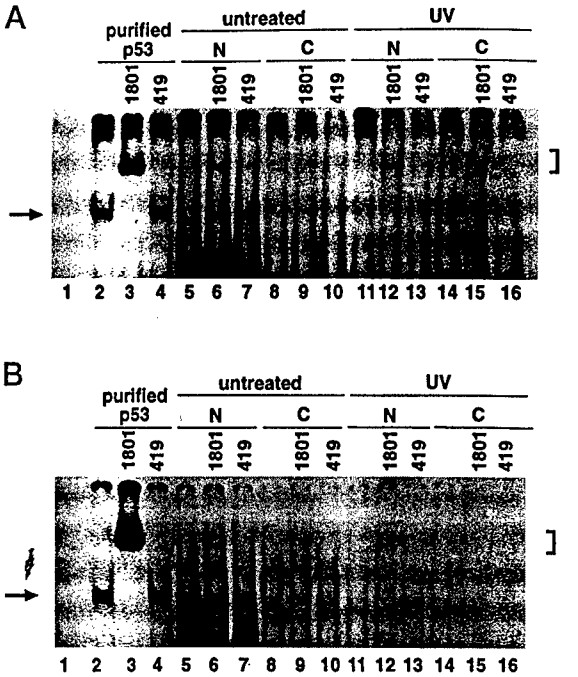

FIG. 9. **p53 in nuclear extracts of untreated WI38 or MCF10F cells binds to DNA in an electrophoretic mobility shift assay.** Nuclear and cytosolic extracts were prepared from WI38 (*A*) or MCF10F (*B*) cells that were untreated (*lanes 5–10*) or 24 h after treatment with 50 J/m^2 of ultraviolet light (*lanes 11–16*). Electrophoretic mobility shift assay was performed as described under "Experimental Procedures." 1 ng of radiolabeled probe (BB.9) was incubated in the absence of protein (*lane 1*), or in the presence of 0.5 μg of human purified human p53 (*lanes 2–4*) or the appropriate amount of nuclear (*lanes 5–7* and *11–13*) or cytosolic (*lanes 8–10* and *14–16*) extracts as indicated. The level of p53 loaded in cellular extracts was normalized by adjusting the total protein. Incubations were performed either with no addition (*lanes 1, 2, 5, 8, 10,* and *14*), or in the presence of either 1801 (anti-p53 antibody, *lanes 3, 6, 9, 12,* and *15*) or 419 (anti-SV40 large T antigen, *lanes 4, 7, 10, 13,* and *16*). The *arrow* to the *left* indicates the position of the p53-DNA complex, and the *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex.

phoretic mobility shift assays (Figs. 7–9). Although untreated extracts from either MCF7, MCF10F, or WI38 cells contain a DNA-binding protein other than p53 which is capable of shifting a specific radiolabeled probe representing a p53 consensus binding site, experiments in the presence of a p53-specific monoclonal antibody 1801 clearly demonstrated that p53 in these extracts could bind to DNA in a sequence-specific manner (Figs. 7–9). Taken together, these results demonstrate that endogenous p53 in untreated, proliferating cells is capable of binding DNA and activating transcription. Thus, p53 is implicated as playing a role in constitutive expression of a particular target gene, that of the cyclin-dependent kinase inhibitor p21, in proliferating cells in the absence of treatment with DNA-damaging agents.

Attempts to demonstrate the ability of p53 from nuclear extracts to bind DNA have often relied on the use of monoclonal antibody 421. The epitope for 421 is located in the carboxyl end of p53, a region that has been suggested to have a negative effect on the sequence-specific DNA binding of p53 (10–15). Studies *in vitro* have demonstrated that 421 can stimulate the binding of wild-type p53 and in some cases can activate select tumor-derived mutant p53 proteins that are incapable of binding to DNA in the absence of antibody (10–12, 14, 47). In the electrophoretic mobility shift analyses performed here, care was taken to avoid the use of monoclonal antibody 421 for these reasons. The presence of a BB.9-binding protein that is not p53 in the untreated extracts made it necessary to supershift gel shift complexes containing p53 in order to detect the complex of p53 with the probe (Figs. 7–9). Use was made of the antibody 1801 that has an epitope on p53 near the amino-terminal end of the protein (28). Studies have demonstrated that in contrast to 421, 1801 does not restore DNA binding activity to mutant p53 proteins (48). It does, however, exert an enhancing effect on the ability of p53 to bind to DNA, but this is due to the ability of 1801 to stabilize p53 against thermal denaturation that occurs during the incubations that are performed to detect specific DNA binding (48, 49). Hence, it is unlikely that 1801 is conferring on the p53 in untreated cell extracts an ability to bind to DNA that this p53 would not otherwise have. Thus, the supershifted complexes produced by incubation with 1801 do indeed reflect the ability of endogenous p53 in the cell to interact in a specific manner with DNA.

Studies utilizing mice that have been homozygously deleted for p53 have shown that the majority of tissues express p21 in a p53-independent manner. Only in the spleen was there substantial differences in p21 expression between p53-null and p53-expressing animals (24). These results indicate that in addition to the p53-dependent mechanism demonstrated here, there must also be p53-independent mechanisms for the regulation of basal levels of p21 expression.

Nevertheless, treatment of cells with DNA-damaging agents clearly inhibits cellular proliferation and involves an increase

in p21 expression that is p53-dependent (1–3). It is reasonable to expect that cells growing *in vitro* experience a low level of oxidative DNA damage, and there may be damage resulting from errors during DNA synthesis. This low level of DNA damage may be responsible for activation of a subset of the p53 protein in the cell leading to transcriptional activation of particular target genes at a low level. Indeed it is likely that cells *in vivo* are subjected to similar low levels of DNA damage. Thus, the results presented here do not necessarily contradict the notion that upon DNA damage, p53 may, in fact, be modified in some way to increase its ability to bind DNA and transcriptionally activate target genes. Post-translational modification of p53 upon DNA damage of cells has been documented, and some studies suggest that this modification may be necessary to achieve the full induction of p53 target gene expression that is seen after treatment with DNA-damaging agents (18, 19).

The human gene for thrombospondin-1 has previously been identified as a target for transcriptional activation by p53 (50). Studies leading to this observation demonstrated that fibroblasts from early passage cells obtained from Li-Fraumeni patients constitutively expressed thrombospondin-1, but later passage cells that had lost expression of p53 no longer secreted thrombospondin-1. Transfection studies demonstrated that the thrombospondin-1 promoter was a target for transcriptional activation by p53, although a specific binding site for p53 in this promoter has yet to be identified (50). As thrombospondin-1 has anti-angiogenic activity, the observation that its constitutive expression is p53-dependent is consistent with the role of p53 as a tumor suppressor. The experiments reported here with the p21 promoter confirm the ability of p53 to transcriptionally regulate constitutive expression of particular target genes in proliferating cells, thereby suggesting a mechanism that is consistent with the report of p53-dependent expression of thrombospondin-1 in proliferating human fibroblasts (50).

Chen *et al.* (27) demonstrated that expression of wild-type but not mutant p53 in Saos-2 cells by retroviral infection will inhibit the ability of these cells to grow in soft agar and grow as tumors in nude mice. Similar results were obtained by retroviral infection of a p53 null peripheral neuroepithelioma cell line (51). As with the studies reported here (Fig. 2 and Table I), the level of p53 that was expressed in the cells in both these studies was sufficiently low to allow the cells to continue to proliferate albeit at a slower rate than the parent cell lines (27, 51). The implication of these observations is that this low level of p53 is capable of suppressing the oncogenic phenotype in these cells suggesting that the ability of p53 to transcriptionally regulate constitutive expression of select target genes may, therefore, play a role in its ability to function as a tumor suppressor. The increased tumorigenicity that results from the loss of basal expression of these p53-dependent targets would then contribute to the selective pressure for the loss of wild-type p53 function in human tumors.

Acknowledgments—We thank Michael Datto and Xiao-Fan Wang (Duke University) for the *p21* promoter reporter constructs; Xiangwei Wu (Mount Sinai School of Medicine) for pCMV-hdm2 and the p21 cDNA probe; Rafael Mira-y-Lopez (Mount Sinai School of Medicine) for MCF7 and MCF10F cells; Bert Vogelstein (Johns Hopkins University) for pCMV-p53$^{Ala\text{-}143}$; and Wen-Hwa Lee (University of Texas) for the packaging cell lines expressing the recombinant retroviruses. The following members of the Manfredi laboratory are thanked for their help and support: Lois Resnick-Silverman, Selvon St. Clair, and Edward Thornborrow.

REFERENCES

1. Gottlieb, T. M., and Oren, M. (1996) *Biochim. Biophys. Acta* **1287,** 77–102
2. Ko, L. J., and Prives, C. (1996) *Genes Dev.* **10,** 1054–1072
3. Levine, A. J. (1997) *Cell* **88,** 323–331
4. Sugrue, M. M., Shin, D. Y., Lee, S. W., and Aaronson, S. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9648–9653
5. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K., and Vogelstein, B. (1993) *Cell* **75,** 817–825
6. Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995) *Nature* **377,** 552–557
7. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995) *Cell* **82,** 675–684
8. el-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., Hamilton, S. R., Kinzler, K. W., and Vogelstein, B. (1995) *Cancer Res.* **55,** 2910–2919
9. Gu, W., and Roeder, R. G. (1997) *Cell* **90,** 595–606
10. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992) *Mol. Cell. Biol.* **12,** 2866–2871
11. Halazonetis, T. D., Davis, L. J., and Kandil, A. N. (1993) *EMBO J.* **12,** 1021–1028
12. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71,** 875–886
13. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83,** 237–245
14. Mundt, M., Hupp, T., Fritsche, M., Merkle, C., Hansen, S., Lane, D., and Groner, B. (1997) *Oncogene* **15,** 237–244
15. Takenaka, I., Morin, F., Seizinger, B. R., and Kley, N. (1995) *J. Biol. Chem.* **270,** 5405–5411
16. Abarzua, P., LoSardo, J. E., Gubler, M. L., and Neri, A. (1995) *Cancer Res.* **55,** 3490–3494
17. Hupp, T. R., and Lane, D. P. (1995) *J. Biol. Chem.* **270,** 18165–18174
18. Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E., and Kastan, M. B. (1997) *Genes Dev.* **11,** 3471–3481
19. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) *Cell* **91,** 325–334
20. Xiong, Y., Zhang, H., and Beach, D. (1993) *Genes Dev.* **7,** 1572–1583
21. Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E., and Givol, D. (1994) *Cancer Res.* **54,** 3391–3395
22. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704
23. Weinberg, W. C., Azzoli, C. G., Kadiwar, N., and Yuspa, S. H. (1994) *Cancer Res.* **54,** 5584–5592
24. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995) *Genes Dev.* **9,** 935–944
25. Datto, M. B., Yu, Y., and Wang, X.-F. (1995) *J. Biol. Chem.* **270,** 28623–28628
26. Friedlander, P., Haupt, Y., Prives, C., and Oren, M. (1996) *Mol. Cell. Biol.* **16,** 4961–4971
27. Chen, P. L., Chen, Y. M., Bookstein, R., and Lee, W. H. (1990) *Science* **250,** 1576–1580
28. Banks, L., Matlewshki, G., and Crawford, L. (1986) *Eur. J. Biochem.* **159,** 529–534
29. Harlow, E., Crawford, L. V., Pim, D. C., and Williamson, N. M. (1981) *J. Virol.* **39,** 861–869
30. Tang, H., Davis, M. A., Strickfaden, S. M., Maybaum, J., and Lawrence, T. S. (1994) *Radiat. Res.* **138,** S109–S112
31. Graeber, T. G., Peterson, J. F., Tsai, M., Monica, K., Fornace, A. J., and Giaccia, A. J. (1994) *Mol. Cell. Biol.* **14,** 6264–6277
32. Ramirez, V. D., Gautron, J. P., Epelbaum, J., Pattou, E., Zamora, A., and Kordon, C. (1975) *Mol. Cell. Endocrinol.* **3,** 339–350
33. Johnson, P., Gray, D., Mowat, M., and Benchimol, S. (1991) *Mol. Cell. Biol.* **11,** 1–11
34. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B., and Friend, S. (1990) *Mol. Cell. Biol.* **10,** 5772–5781
35. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. (1990) *Science* **249,** 912–915
36. Finlay, C. A., Hinds, P. W., and Levine, A. J. (1989) *Cell* **57,** 1083–1093
37. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8763–8767
38. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993) *Nature* **362,** 857–860
39. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) *Cell* **69,** 1237–1245
40. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387,** 299–303
41. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387,** 296–299
42. Guillot, C., Falette, N., Paperin, M. P., Courtois, S., Gentil-Perret, A., Treilleux, I., Ozturk, M., and Puisieux, A. (1997) *Oncogene* **14,** 45–52
43. Fan, S., Smith, M. L., Rivet, D. J. I., Duba, D., Zhan, Q., Kohn, K. W., Fornace, A. J. J., and O'Connor, P. M. (1995) *Cancer Res.* **55,** 1649–1654
44. Casey, G., Lo, H. M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. (1991) *Oncogene* **6,** 1791–1797
45. Hinds, P. W., Finlay, C. A., Quartin, R. S., Baker, S. J., Fearon, E. R., Vogelstein, B., and Levine, A. J. (1990) *Cell Growth Differ.* **1,** 571–580
46. Isaacs, J. S., Chiao, C., Merrick, B. A., Selkirk, J. K., Barrett, J. C., and Weissman, B. E. (1997) *Cancer Res.* **57,** 2986–2992
47. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1993) *Nucleic Acids Res.* **21,** 3167–3174
48. Friedlander, P., Legros, Y., Soussi, T., and Prives, C. (1996) *J. Biol. Chem.* **271,** 25468–25478
49. Liao, K., and Lane, D. P. (1995) *J. Biol. Chem.* **270,** 12123–12132
50. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. (1994) *Science* **265,** 1582–1584
51. Chen, Y. M., Chen, P. L., Arnaiz, N., Goodrich, D., and Lee, W. H. (1991) *Oncogene* **6,** 1799–1805
52. Resnick-Silverman, L., St. Clair, S., Maurer, M., Zhao, K., and Manfredi, J. J. (1998) *Genes Dev.* **12,** 2102–2107